The presence of faecal pollution, and potential plant pathogens associated with onion production, in the Lower Vaal River

by

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SUMMARY

Industrial and sewage pollution of the Vaal River pose a threat to sustainable agriculture along the Lower Vaal River in the Northern Cape of South Africa. In this region, the Vaal River is used to irrigate onion fields via centre-pivot irrigation systems. However, irrigation water originating from rivers may, in addition to human pathogens, contain waterborne plant pathogens that are washed into river systems by agricultural surface runoff, posing a threat to crop production. Thus, the goals of this study were firstly to screen for both faecal contamination in the Lower Vaal River, as well as the onion pathogen *Fusarium oxysporum* f. sp. *cepae* (*Focep*). Subsequently, the efficacy of a calcium hypochlorite containing disinfectant, i.e. „HTH® Super Shock It", was determined to remove coliform bacteria and *Fusarium* spores from the river water within 16 s. This is the contact time allowed for a disinfectant when water is treated at the hub of a centre-pivot irrigation system, before it is dispersed via the first sprinklers in a typical centre-pivot irrigation system.

Surface water samples, as well as water from various pump stations used for irrigation, were collected over a period of three years at sampling sites along a 159 km stretch of the Vaal River. Sample analyses revealed that faecal coliforms were always present (7.19×10^5 CFU/100 ml). Also, 59 *Fusarium* isolates were obtained from the water, as well as eight *Fusarium* isolates from onion bulbs that were cultivated in fields irrigated by water from the river. Molecular identification revealed that the isolates belonged to four *Fusarium* species, i.e. *Fusarium brachygibbosum*, *Fusarium incarnatum-equiseti*, *Fusarium solani* and *Fusarium oxysporum*, the latter being the dominant species represented by 52 isolates. The pathogenicity of the *Fusarium* isolates was determined against onion bulbs (Lombardi cultivar), and it was found that none of the water isolates caused basal rot and were therefore not representatives of *Focep*. However, four *F. oxysporum* isolates obtained from the onion bulbs were found to be *Focep* belonging to the vegetative compatibility group (VCG) 0425, previously known to be prevalent in the Western Cape Province. Despite the fact that no pathogenicity toward onion could be confirmed among the waterborne *Fusarium* isolates, subsequent screening for virulence factors, i.e. *SIX* (secreted in xylem) genes, revealed the presence of the *SIX7* gene in some isolates. These isolates may therefore be potentially pathogenic to crops other than onion cultivated in the Lower Vaal region.

A concentration of 1.50 mg/L „HTH® Super Shock It" was discovered to effectively remove 100% of faecal coliforms within 16 s from the water, while *Fusarium* spores were removed at 7.50 mg/L after 3600 s. Thus, while the disinfectant may be ineffective at removing fungal plant pathogens from a centre-pivot irrigation system within the required time, the results indicate that it will remove coliform bacteria from the water before it is dispersed onto crops.

SAMEVATTING

Industriële en rioolbesoedeling van die Vaalrivier stel volhoubare landbou langs die Laer Vaalrivier in die Noord-Kaap in gevaar. In hierdie streek word die Vaalrivier gebruik om uielande deur middel van spilpuntbesproeiing nat te lei. Besproeiingswater uit riviere mag, saam met menslike patogene, ook watergedraagde plantpatogene bevat wat in die riviersisteme beland deur oppervlakte-afloop van boerderye, en dus „n gevaar inhou vir oes-produksie. Die oorhoofse doelwitte van hierdie studie was dus eerstens om die vlak van fekale kontaminasie van die Laer Vaalrivier te bepaal, en ook om vas te stel of die uie-patogeen *Fusarium oxysporum* f. sp. *cepae* (*Focep*) in die rivier voorkom. Die doeltreffendheid van „n kalsium-hipochlorietbevattende ontsmettingsmiddel, naamlik „HTH® Super Shock It", om kolivorme bakterieë en *Fusarium* spore binne 16 s uit die rivierwater te verwyder, is gevolglik bepaal. Dit is die kontaktyd wat vir „n ontsmettingsmiddel toegelaat word wanneer water by die spil van „n spilpuntbesproeiingstelsel behandel word, voor dit deur die eerste sproeiers van „n tipiese spilpuntbesproeiingstelsel versprei word.

Oppervlakwatermonsters, asook water uit verskeie pompstasies wat vir besproeiing gebruik word, is oor 'n tydperk van drie jaar by monsternemingsterreine binne 'n 159 km gedeelte van die Vaalrivier versamel. Monsteranalises het getoon dat fekale kolivorme altyd teenwoordig was (7.19 x 10^5 CFU/100 ml). Daar is ook 59 *Fusarium* isolate uit die water gekry, asook agt *Fusarium* isolate van uiebolle wat verbou is in lande wat met water uit die rivier besproei is. Molekulêre indentifikasie het getoon dat die isolate tot vier *Fusarium* spesies, naamlik *Fusarium brachygibbosum*, *Fusarium incarnatum-equiseti*, *Fusarium solani* en *Fusarium oxysporum* behoort, waarvan laasgenoemde die dominante spesies is en deur 52 van die isolate verteenwoordig word. Die patogenisiteit van die *Fusarium* isolate teenoor uiebolle (Lombardi kultivar) is bepaal, en daar is gevind dat nie een van die waterisolate wortelvrot veroorsaak het nie, en dus nie verteenwoordigers van *Focep* is nie. Vier *F. oxysporum* isolate wat van uiebolle in die lande verkry is, is egter gevind om *Focep* te wees wat tot die vegetatiewe verenigbare groep (VCG) 0425 behoort, wat voorheen bekend was om in die Wes-Kaap voor te kom. Ten spyte van die feit dat geen patogenisiteit (teenoor uie) onder die watergedraagde *Fusarium* isolate bevestig kon word nie, het daaropvolgende siftings vir virulensiefaktore, naamlik *SIX* ("secreted in xylem") gene, die teenwoordigheid van die *SIX7*-geen in sommige isolate getoon. Hierdie isolate mag dus potensieel patogenies wees vir gewasse anders as uie wat in die Laer Vaal-gebied verbou word.

„n Konsentrasie van 1.50 mg/L „HTH® Super Shock It" is benodig om 100% fekale kolivorme effektief binne 16 s uit die water te verwyder, terwyl *Fusarium* spore verwyder is teen 7.50 mg/L na 3600 s. Terwyl die ontsmettingsmiddel dus oneffektief is om fungus plantpatogene binne die verlangde tydperk uit die spilpuntbesproeiingstels te verwyder, toon resultate dat dit wel kolivorme bakterieë uit die water sal verwyder voor dit oor die gewasse versprei kan word.

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"*Let go your earthly tether. Enter the void. Empty, and become wind.*" - Guru Laghima

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1.1 Introduction

Primary agriculture is seen as an important sector in South Africa's economy, despite its small share (R60 billion) in the total gross domestic product (GDP) (DAFF, 2013). It is a significant provider of employment, particularly in rural areas, and the most important stipendiary of foreign exchange. The outputs of agriculture function as intermediate products with about 70% exchanged in the manufacturing sector. The agricultural sector has grown by an average of 11.8% per annum since 1970. Over the same period however, the total economy grew 14.9% per annum, resulting in a drop in agriculture's share of the GDP from 7.10% in 1970 to 2.50% in 2010.

According to the Population Division of the United Nations, the current world population of 7 billion will surpass 9 billion by 2050 (Department of Economic and Social Affairs, 2011). Coupled with global climate change and extreme weather events becoming more frequent, an increase in population will result in enormous strain on natural resources. Consequently, the ability to provide adequate nutritious food through sustainable means may be compromised (Rothamsted Research, 2012). Nevertheless, global food production will have to increase with 40% by 2030 and a further 70% by 2050 (OECD-FAO, 2009). Additionally energy and water demand could double in the not-so-distant future (Foresight, 2011). Another challenge for higher food production is waterborne human pathogens dispersed via polluted ground water, surface water and human wastewater onto irrigated crops (Steele & Odumeru, 2004; Steele *et al*., 2005; Alsanius *et al*., 2010; Ijabadeniyi *et al*., 2011; Gottschall *et al*., 2013). Generally, water quality is assessed by the presence of faecal contamination, which closely relates to the occurrence of microorganisms that are pathogenic to humans (Steele & Odumeru 2004; Steele *et al*., 2005). However, waterborne plant pathogens, such as the filamentous fungus *Fusarium oxysporum,* may also enter agricultural land via irrigation water thereby posing a threat to sustainable crop production (Bucheli *et al*., 2008; Summerell *et al*., 2010; Van Wyk *et al*., 2012).

One of South Africa's most important rivers, is the Vaal River. The water thereof, is used for industry and agricultural purposes. Unfortunately the water quality was found to be deteriorating due to polluted wastewater originating mostly from urban and industrial developments upstream from its lower regions, where the river is largely used for agricultural purposes (DWAF, 2009; DWAF, 2011). Here, on the banks of the Lower Vaal River in the Northern Cape, industrial and sewage pollution may pose a threat to sustainable agriculture (Le Roux *et al*., 2007). In this region, where one of South Africa's major onion production areas is situated (DAFF, 2010), the Vaal River is used for intensive irrigation practices. Thus, the overall goals of this study were firstly to screen for faecal contamination in the Lower Vaal, as well as to determine whether the river contains *F. oxysporum* strains that are pathogenic to onions. Subsequently, the efficacy of a calcium hypochlorite containing disinfectant to remove faecal indicator bacteria and *Fusarium* spores from the river water was determined.

1.2 Water quality of rivers in South Africa

It is known that pollution is on the rise in South African river systems as revealed by a number of studies conducted to assess quality of important water sources in this country (Chutter, 1971; Genthe, 1997; Muller *et al.*, 2001; Jamieson *et al.*, 2002; Obi *et al.*, 2004; Jackson *et al.*, 2009a; Jackson *et al*., 2009b; Wepener *et al.*, 2011). Situated within a semi-arid portion of the world, South Africa is characterised by high seasonal variation in rainfall and runoff, as well as high evaporation rates. This causes stream flow to be moderately low for a majority of the year, with only sporadic high flows occurring seasonally.

Furthermore, stress is being exerted onto water resources through population expansion and increased industrial and urbanisation activities (Schutte & Pretorius, 1997; Ochse, 2007). Worryingly, it has been reported that South Africa has no more surplus water and has therefore, lost its dilution capacity to reduce pollutant levels (Turton, 2008). Due to a scarcity of local fresh water, the use of river water for irrigation, amongst other uses (industrial, mining, and power generation, domestic and municipal use) is widely practiced in developing countries with South Africa being no exception, however, potential risks are associated with its use (Schutte & Pretorius, 1997; Gemmell & Schmidt, 2011). A study conducted by Gemmell & Schmidt (2011) has revealed that feacal matter entered the Baynespruit River in Sobantu. In addition, Obi *et al.* (2002) found water sources in the Venda region of South Africa to be of poor microbial quality. Their results showed that indicator organisms exceeded the maximum limits prescribed by the Department of Water Affairs and Forestry (DWAF) of South Africa (DWAF, 1996). The water sources studied by Obi *et al.* (2002) included the Lebuvu River, as well as the Vuwani, Mutale, Ngwedi, Tshinane, Makonde, Mutshindudi and Mudaswali Rivers. Numerous studies also showed a decline in water quality of rivers in the Western Cape. These rivers, amongst others, include the Plankenburg and Diep River (Jackson *et al*., 2009b). It is therefore evident from the above, that river pollution is a widespread phenomenon in South Africa.

1.3 The Vaal River

The Vaal River is the most highly utilised river in the country (DWAF, 2009; DWAF, 2011), making it a strategically important water resource for sustainable economic development in South Africa. The river arises on the High Veld plateau of Mpumalanga and eastern Free State, an area of undulating plains, primarily formed by shales and sandstones of the Karoo system (Butzer, 1973). At the end of the Vaal River‘s 1,350 km course, the valley broadens out onto the Middle Veld, continuing its flow past Willowbank, Riverton, west into Barkley West and finally, it joins the Orange River at 957 m elevation near Douglas in the Northern Cape.

The Vaal River is divided into three water management areas (WMA), indicated in Figure 1.1**;** the Upper, Middle and Lower WMAs, each having individual catchment and sub-catchment systems (DWAF, 2009; DWAF, 2011). The catchment areas stretch from the northeast at Ermelo to the northwest at Vryburg, toward Douglas in the southwest, and east at Harrismith. Serving as a conduit the Vaal River transfers water amongst the three WMAs. Essentially four major storage dams in the Vaal River Basin comprise the main Vaal System; these include the Grootdraai Dam, Sterkfontein Dam, Vaal Dam, and Bloemhof Dam. With the exception of the Sterkfontein Dam, situated on the Wilge River tributary, the dams are situated on the main stem of the Vaal River.

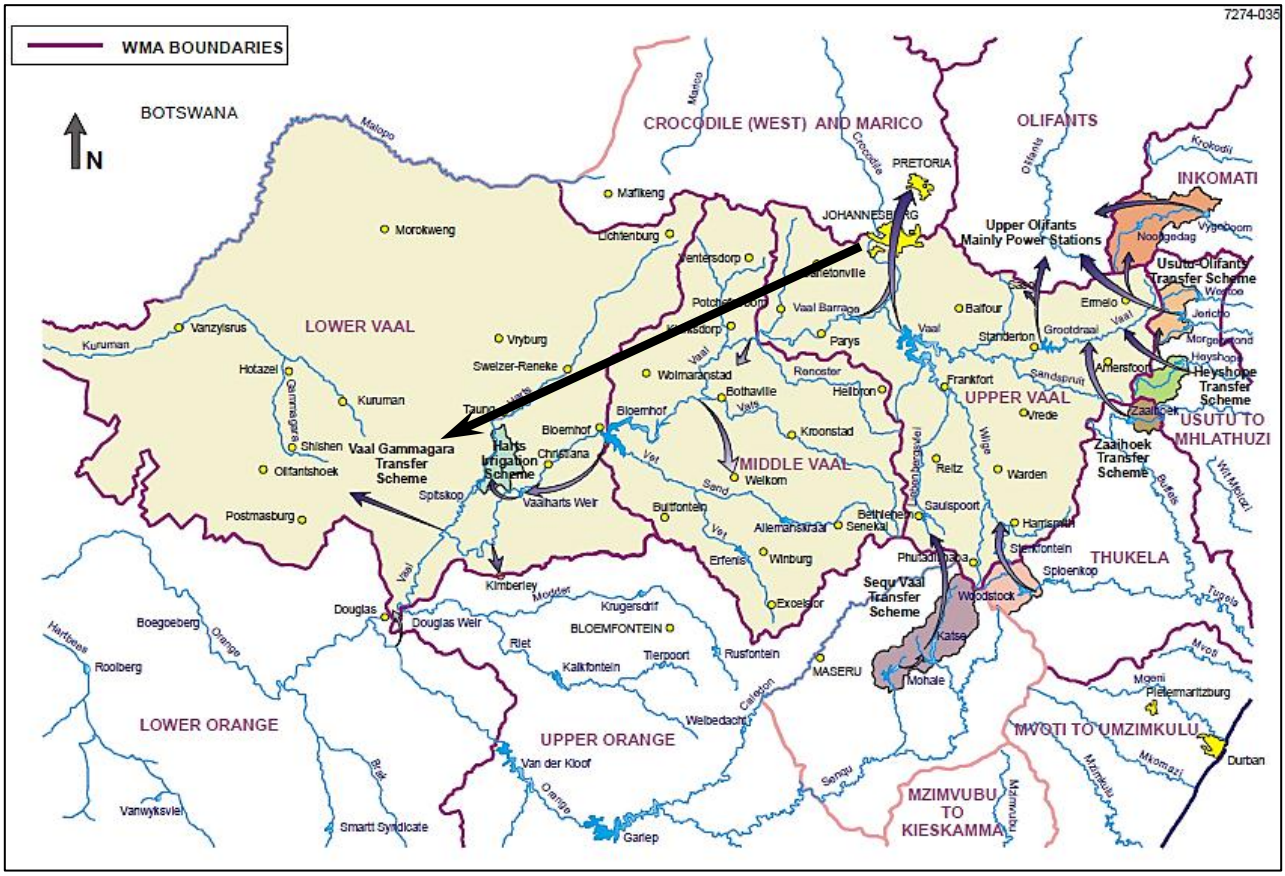

Figure 1.1. The Vaal River water management areas (adapted from DWAF, 2009). The black arrow indicates the Vaal River main flow, starting from the Upper Vaal flowing through the Middle Vaal and exiting at the Lower Vaal. The purple arrows show associated water transfer schemes.

1.3.1 The Upper Vaal WMA

Located in the centre of the country, the Upper Vaal WMA covers a 55 562 km^2 catchment area including parts of Gauteng, Free State, Mpumalanga and the North-West provinces (DWAF, 2009). This uppermost WMA of the Vaal River system includes the dams, Grootdraai and Vaal Dam (Figure 1.1). The Vaal River and Wilge River flow into the Vaal Dam upstream of the Vaal River

Barrage (Stephenson, 2002). Populated with an estimated 18.1 million people (2009) it is the most populated region in the country (DWAF, 2004b; Statistics South Africa, 2010). In the northern and western parts of this WMA land use is characterised by expansive urban, mining, and industrial areas (DWAF, 2009). Other land uses in the area relate to dry land agriculture along with livestock farming including, intensive irrigation practices occurring along the main river.

1.3.2 The Middle Vaal WMA

Forming part of the Orange River watercourse, the Middle Vaal WMA covers a catchment area of 52 563 km^2, which includes parts of the Free State and North-West provinces (DWAF, 2009). The Vaal River flows westerly through this middle WMA towards Bloemhof Dam and into the Lower Vaal WMA (Figure 1.1). Populated by nearly 4.0 million people (2009), the Middle Vaal WMA is rural in nature. Land use is characterised by extensive livestock farming, dry land agriculture, as well as irrigation farming. While these agricultural activities are remaining relatively stable, major gold mining operations in the region are on the decline (DWAF, 2004c; DWAF, 2009; Statistics South Africa, 2010).

1.3.3 The Lower Vaal WMA

Situated in the north-western part of the country the Lower Vaal WMA forms part of the Orange River watercourse, spanning a catchment area of 133 354 km^2 (DWAF, 2009). It comprises of areas within the Northern Cape and North-West Provinces, as well as a small part of the Free State Province (Figure 1.1). The only major river of this WMA is the Vaal River that flows westerly with the Orange River from Bloemhof Dam towards the confluence. The biggest part of the Lower Vaal WMA falls within the Molopo River catchment, which is also a tributary of the Orange River. The population of the Lower Vaal WMA is approximately 4.5 million (2009). Land use in this area is characterised by extensive livestock farming as the main activity and, in the north eastern part of the WMA, large scale dry land cultivation takes place, while intensive irrigation practices occurs along the main river in the south of this WMA (DWAF, 2004c; Statistics South Africa, 2010).

1.4 Water quality of the Vaal River

The DWAF, as well as three major water boards namely, Rand Water, Midvaal Water, and Sedibeng Water conducted a 10-year study, of water quality in the Vaal River (DWAF 2009). The study uncovered some issues related to the whole length of the Vaal River, while other problems are more localised, however the greatest impact on water usage has been the increase in salinity (and related macro ions).

The rise in total dissolved salts (TDS) and associated rise in chloride and sulphate levels may have major consequences on domestic, industrial, and agricultural water use in the region (DWAF, 2009). Microbiological pollutants and elevated levels of certain metals (localised problems) are also an emerging concern. Eutrophication is another water quality concern in the Vaal River System, resulting in algal blooms and water hyacinth growth, however good quality water occurs in the upper catchment areas.

Areas of concern were found to be the Vaal Barrage, Middle Vaal River, and Lower Vaal River downstream of the Harts River confluence, which has high TDS levels. More recently, the degradation of natural ecosystems was recognised in most of the Vaal River‘s main sections and tributaries (DWAF, 2011). This is a concern to farmers along the river since the quality of the water they use for irrigation purposes might affect their harvests.

1.4.1 Upper Vaal

A meeting on water quality held on 20 November 2007 in South Africa‘s Gauteng province between non-governmental organisation (NGO) supporters and local water management officials, revealed the extent of the pollution of the Vaal River (Tempelhoff, 2009; Botes, 2007; Seale, 2007). Findings of independent laboratory tests showed dangerously high levels of faecal pollution in the Vaal River Barrage. It was reported that large volumes of sewage end up in the Vaal River due to expanding urban areas, as well as sewage return flow from municipal wastewater works (DWAF, 2009; Tempelhoff, 2009). Wastewater discharged into the catchment by a number of municipalities ends up in the Vaal River via the tributaries. As a result of these activities the threat of sewage pollution was recognised in addition to the impact of extensive industrial and mining developments in the Upper Vaal WMA (Tempelhoff, 2009).

1.4.2 Middle Vaal

Water entering the Middle Vaal WMA from the Upper Vaal WMA, brings with it a large amount of urban, industrial and mining return flows from areas within the Upper Vaal WMA (DWAF, 2009). These areas are highly industrialised and urbanised carrying high salinity levels and nutrient concentrations. Fresh water from the Vaal Dam is used to dilute the high levels of salinity and insure that acceptable quality water reaches the Middle Vaal WMA. Similar to the situation in the Upper Vaal WMA the urban areas contribute to sewage return flows that carry significant pollution loads. The return flows end up directly in the Vaal River after being discharged from a number of municipalities into the catchment. In addition, return flows from the mining industry contribute to water pollution in the Middle Vaal WMA.

1.4.3 Lower Vaal

The main source of water in the Lower Vaal WMA is the surface flow of the Vaal River that originates from the Upper and Middle Vaal (DWAF, 2009). This water is mostly used for urban, agricultural and mining purposes. About 80% of the water is used for intensive irrigation practices at Vaalharts and other locations along the Vaal River, with over 90% of required water sourced through releases from the Bloemhof Dam and Upper Vaal WMA. It must also be noted that water from the Vaalharts weir, on the Vaal River, is transferred in large quantities to supply the Vaalharts irrigation scheme in the Harts River catchment (DWAF, 2009). Irrigation return flows are generated by this irrigation scheme and enter the Harts River upstream from Spitskop Dam. Return flows bring forth salanity and nutrients to the Harts River. In addition, some municipalities discharge wastewater either directly into the Vaal River or into the catchment via the Harts River. It was previously stated that water quality of the rivers in the WMA is of acceptable quality, despite high turbidity levels that are exhibited at times however; the water quality of the Lower Vaal River water has deteriorated over the past decades and is expected to do so even further. This deteriorating water quality was atributed to polluted wastewater, originating from Gauteng, ending up in the Lower Vaal River. Here, irrigating with poor quality water may result in increased soil salinity and a threat to crop production (Le Roux *et al*., 2007). In addition, agricultural surface runoff flowing into rivers may contain waterborne plant pathogens, which may pose an additional threat to production when introduced to crops via irrigation (Bucheli *et al.,* 2008; Summerell *et al.*, 2010; Van Wyk *et al*., 2012). Intensive irrigation practices, utilising centre-pivot technology, occur along the main river in the south of the Lower Vaal WMA where one of South Africa‘s major onion production areas is situated (DAFF, 2010). This large-scale onion cultivation may therefore be at risk, since waterborne plant pathogens, associated with onion production, may occur in the Lower Vaal River.

1.5 Factors affecting water quality in rivers used for irrigation

Water is essential for the sustainment of life, it should be safe, accessible, in adequate amounts and available to all (WHO, 2008). The term water quality encompasses the physical, biological, chemical, and aesthetic properties of water. This determines the fitness of water for various uses and for protection of aquatic ecosystems (DWAF, 1996). Good quality water should have no odour and be free of any taste. Consumers evaluating the quality and acceptability of water, use these criteria. Constituents that affect the appearance, odour or taste of water are pollutants of chemical, microbial and biological origin (WHO, 2008).

1.5.1 Chemical pollutants

Increased populations, and deforestation of land for agricultural and urban purposes, has led to the degradation of surface and groundwater quality, by chemical pollution through fertilisers and pesticides (Novotny, 1999). Agricultural activities cause chemical contamination of river systems; such as excess fertilisers, pesticides, and manure runoff from agricultural land into nearby rivers. Chemicals that are a health risk include aluminium, ammonia, chloride, and copper, to mention a few. These chemical pollutants were an unobserved health risk before the 1950s, when organic fertilisers were used on a relatively small scale on farms, leaving waste products to be easily assimilated by receiving water bodies and soils (Novotny, 1999). Farming post 1950s however, saw a worldwide shift in the agricultural sector, with intensive farming operations utilising large quantities of chemical fertilisers and pesticides to increase yields. Chemical pollutants can also originate from industrial sources such as mining industries, sewage plants or urban runoff that end up in river systems (WHO, 2008).

1.5.2 Microbial pollutants

Biological contamination of water is caused by an over proliferation of certain organisms such as, invertebrate animals, as well as microorganisms namely, actinomycetes, algae, cyanobacteria, fungi and iron bacteria (WHO, 2008; Gemmell & Schmidt, 2011). These microorganisms occurring in the river water can be disseminated via irrigation onto fresh produce. It was previously demonstrated that potential links exist between the quality of river water used for irrigation and the microbiological quality of fresh produce (WHO, 2008; Gemmell & Schmidt, 2011). The microbial contaminants associated with low quality water are generally perceived to be pathogens of public health concern. These pathogens are usually bacteria, viruses and protozoa (WHO, 2008).

1.5.2.1 Bacteria

It is known that pathogenic bacteria are responsible for animal and human diseases, all of which are generally transmitted via direct contact with an infected host or by ingestion of contaminated food or water (Schroeder & Wuertz, 2003). The most prominent waterborne bacterial pathogens, as well as their associated diseases are listed in Table 1.1. The bacteria are all Gram-negative and belong to the phylum *Proteobacteria,* while the most common disease among humans reported for these bacteria is gastroenteritis (Payment, 1991; Payment, 2003). The most common cause of gastroenteritis in humans in developed countries was found to be infection by proteobacteria belonging to the genus *Salmonella,* which are also the most predominant pathogenic bacteria found in wastewater (Baggesen *et al*., 2000; Bell & Kyriakides, 2002; Bitton, 2005).

The genus *Salmonella* is subdivided into two species, *Salmonella bongori* and *Salmonella enterica* (Levantesi *et al*., 2012). *Salmonella enterica* is further grouped into six subspecies i.e., *arizonae*, *diarizonae*, *enterica*, *houtenae*, *indica* and *salamae*, of which *S. enterica* subspecies *enterica* was found to be the most prevalent among mammals including man. *Salmonella* strains are also classified into several serovars according to their flagellar and somatic antigens. While more than 2400 serovars have been described, only about 50, all within the subspecies *enterica,* are known to cause infection of warm blooded animals (Popoff, 2001). It must be noted that *Salmonella* contaminated waters, used to irrigate and wash crops, have been implicated in a large number of food-borne disease outbreaks across the globe (Levantesi *et al*., 2012).

Table 1.1. Major waterborne bacterial pathogens which affect the gastrointestinal tract (Sobsey & Olson, 1983; Leclerc *et al*., 2002; Levantesi *et al*., 2012).

Bacterial species	Disease	Origin
Campylobacter jejuni	Gastroenteritis	Human/animal faeces
Virulent *Escherichia coli*	Gastroenteritis	Human faeces
Salmonella enterica	Typhoid fever, Paratyphoid fever, Gastroenteritis	Human/animal faeces
Shigella sonnei	Gastroenteritis	Human faeces
Vibrio cholerae	Cholera	Human faeces
Yersinia enterocolitica	Gastroenteritis	Human/animal faeces

Other well-known waterborne human pathogens are bacterial species belonging to the genus *Vibrio* (Vezzulli *et al*., 2013). They are known to be among the most common bacteria to inhabit surface waters globally and are responsible for some of the most severe human and animal infections. Typical illnesses caused by *Vibrio* spp. include fatal acute diarrheal diseases, such as cholera, wound infections, septicemia and gastroenteritis. The most pathogenic species of the genus is *Vibrio cholerae*, which globally accounts for an estimated three million cases of cholera annually and a projected case fatality rate of about 2.4% (Ali *et al*., 2012).

Other important waterborne bacterial pathogens are *Campylobacter jejuni, Shigella sonnei and Yersinia enterocolitica* (Table 1.1). They can readily be found in diverse environments such as animal intestines and faeces, sewage, environmental water sources, agricultural surface water and food samples, and are all known to be enteric pathogens causing bacterial gastrointestinal diseases in humans worldwide (Terzieva & McFeters, 1991; Waage *et al*., 1999; Colles *et al*., 2003; Stabler *et al*., 2013; McDonnell *et al*., 2013).

In addition, of all the bacterial genera, *Escherichia* is the most widely studied genus (Krieg, 1984; Jay, 2005). Representatives of this genus are Gram-negative, non-sporeforming, and facultative-anaerobic bacteria belonging to the family *Enterobacteriaceae*, which in turn belongs to the class Gamma-Proteobacteria. The best-known species is *Escherichia coli,* commonly found in the gastrointestinal tract of mammals, including humans (Reiss, 2006). Although this species is generally perceived as commensal, pathogenic *E. coli* strains do exist. These pathogenic strains are grouped according to their specific virulence traits resulting in diarrhea, as well as the clinical syndrome that is produced during infection. Consequently, there are five common pathogenic groups within *E. coli*; enterotoxigenic, enteropathogenic, enteroaggregative, enteroinvasive and enterohemorrhagic *E. coli* (EHEC), with the latter being predominantly virulent. Importantly, the most common EHEC strain, *E. coli* O157:H7, has been isolated from plant tissue treated with contaminated irrigation water. Solomon and co-workers (2002) previously examined *E. coli* O157:H7 entering lettuce through the root system and migrating towards the edible leaves of the plant. Other researchers also found that this pathogen is introduced to vegetable crops via irrigation water (Erickson *et al.,* 2010; Fonseca *et al.,* 2011).

Due to its presence in the gastrointestinal tract *Escherichia coli* forms part of the group called faecal coliforms, which act as indicators for faecal pollution in water sources, as explained in later sections. However if coliforms cannot be detected in water, other pathogens such as protozoa and enteric viruses may still be present (Grabow, 1996).

1.5.2.2 Viruses

Enteric viruses can be found in water sources such as, leaking sewage or agricultural runoff and pose a health threat to both animals and humans (Fong & Lipp, 2005). More than 100 types of pathogenic viruses are present in animal and human excrement, transmitted via the faecal oral-route (Melnick, 1984). These enteric viruses are specific to their host and cause a range of infections in the gastrointestinal tract. The most commonly studied groups fall under the families *Adenoviridae* (adenoviruses), *Picornaviridae* (polioviruses, enteroviruses, and hepatitis A virus), *Reoviridae* (reoviruses and rotaviruses) and *Caliciviridae* (astroviruses, caliciviruses, noroviruses, and small round-structured viruses) (Fong & Lipp, 2005). Enteric virusses are associated with human diarrheal infections and self-limiting gastroenteritis.

Other infections caused by these viruses include paralysis in immunocompromised individuals, respiratory infections, hepatitis, conjunctivitis, aseptic meningitis, and encephalitis (Kocwa-Haluch, 2001). Some chronic diseases such as myocarditis and insulin-dependent diabetes are also associated with enteric viruses (Kocwa-Haluch, 2001; Griffin *et al*., 2003).

1.5.2.3 Protozoa

Representatives of the genus *Cryptosporidium* are parasitic protozoans and are of a public health concern, causing gastrointestinal diseases (King & Monis, 2007). *Cryptosporidium* oocysts are prevalent in water sources subjected to human and animal faecal contamination and can persist in fresh water for weeks under cool conditions. A study conducted on a livestock farm in the United Kingdom revealed that these oocysts were present in streams throughout the year with the highest amount coinciding with increased animal numbers (Bodley-Tickell *et al*., 2002).

Another waterborne protozoan, the causative agent of giardiasis in humans, is *Giardia*. Seen as a serious waterborne human pathogen since the 1960's, *Giardia's* lifecycle exists in two phases (Wallis *et al*., 1996). Inside the intestine *Giardia* exists in its flagellated form and is able to multiply. Inside the faeces, it exists as thick-walled cysts occurring in elevated numbers. Having robust cysts similar to *Cryptosporidium,* this protozoan can also persist in water for weeks.

Both aforementioned protozoa are significant waterborne pathogens, responsible for causing diarrhoea and nutritional disorders in both humans and animals worldwide (Savioli *et al*., 2006). Morbidity and mortality associated with protozoan infections are high, with more than 58 million cases of childhood protozoal diarrhoea reported per year. More research into the impact of these protozoan species on developing countries are underway, as protozoan parasitic infections form part of the World Health Organisation (WHO) neglected diseases initiative.

1.5.2.4 Fungi

1.5.2.4.1 Waterborne fungal pathogens

Due to the more acute infections caused by bacteria, viruses and protozoan parasites, fungi are seldom mentioned during discussions of waterborne pathogens (Hageskal *et al*., 2009). Nevertheless, fungi are relatively common in water distribution systems, which are known to harbour allergenic, pathogenic, and toxigenic filamentous fungal species belonging to the genera *Aspergillus*, *Alternaria* and *Fusarium.* Many mycotoxin and aflatoxin producing fungi may be present in river water, as was discovered in a study conducted on the Nile River (Hameed *et al*., 2008). These fungi can impact human health since mycotoxins, in low concentrations, might impair intestinal health and immune functions (Antonissen *et al*., 2014).

Also, it is known that waterborne fungi, may pose a possible risk to immunocompromised patients (Hameed *et al*., 2008; Hageskal *et al*., 2009) In addition to filamentous fungal species, various yeast species belonging to the genera *Candida*, *Cryptococcus, Pichia,* and *Rhodotorula,* also occur in aquatic environments in significant numbers (Medeiros *et al*., 2008). Recently, *Candida albicans* was observed in the sewage polluted Plankenburg River, South Africa (Stone *et al*., 2012), and a variety of *Candida* species were found in non-impacted natural lakes as well as nearby polluted river water in south eastern Brazil by Medeiros and co-workers (2008).

Candida albicans is a pathogen causing many forms of diseases, of which some can be life-threatening (Hazen, 1995). This pathogen is able to infect nearly every organ in the body and is the most common yeast isolated from blood, causing nosocomial infections and systemic candidiasis in severely immunocompromised individuals (Hazen, 1995; Ruhnke, 2006). However, other *Candida* species such as, *Candida dubliniensis* and *Candida tropicalis* may also be causative agents of infection, causing renal lesions and fungal masses in different organs (Koga-Ito *et al*., 2010).

However, waterborne fungi may not only impact human health. Representatives of the genera *Aspergillus*, *Alternaria* and *Fusarium* are also known to pose a risk to crops, causing various diseases such as head blight or scab in wheat, red ear rot in maize, basal rot of onion and black rot of olive and citrus, to name a few (Logrieco *et al*., 2003; Schwartz & Mohan, 2008).

1.5.2.4.2 *Fusarium*: Waterborne plant pathogens

The genus *Fusarium* was first described by Link (1809) and was based on the species *Fusarium roseum* (Booth, 1971; de Hoog, 2000). He described the genus as being characterised by fusiform (spindle shaped, swollen in the middle and narrow at the ends) non-septate spores, borne on a stroma (a large irregular mass of vegetative hyphae). Making use of the International Botanical Code, Fries (1821) validated the genus and included it in the order Tuberculariae. During the following century about 1000 *Fusarium* species were described in terms of host associations. Based on a range of morphological features these descriptions were later consolidated by Wollenweber and Reinking (1935) into 65 species (Summerell *et al.*, 2010). Later Snyder and Hansen further reduced the number of species in *Fusarium* to nine (Snyder & Hansen, 1945; Nelson *et al*., 1994). Their system was based primarily on the morphology of the macroconidia and an extensive study of the general nature and variability of *Fusarium* species. Their taxonomic studies were based on extensive single-conidium analysis of *Fusarium* cultures under identical culture conditions. Snyder and Hansen's study on *Fusarium oxysporum* Schlecht. emend. Snyd. & Hans. (section Elegans) formed the basis for their taxonomic system.

This work illustrated the importance of cultural variation in the taxonomy of these fungi, while their studies on *Fusarium solani* (Mart.) Sacc. emend Snyd. & Hans., showed that the variations are inheritable features of these fungi. Today, the genus *Fusarium* is known to harbour some of the most notorious waterborne plant pathogens (Summerell *et al.,* 2010). Globally, these fungi are known to affect agricultural products and may even have an impact on public health. A number of *Fusarium* species produce mycotoxins and others are opportunistic human pathogens, causing diseases such as fusariosis and keratitis (Anaissie, 2001; Chang, 2006).

Important plant diseases caused by *Fusarium* are head blight of wheat and Fusarium wilt of bananas. Globally, *Fusarium* infection leads to enormous losses in crop production, and negatively affects the communities that rely on sustainable crop production (Doidge *et al*., 1954; Ploetz & Pegg, 1997; McMullen *et al*., 1997). Some diseases caused by *Fusarium* spp. toward onions are, damping-off, Basal Rot, Bulb Rot and Pink Root. Infection by this organism leads to devastating losses of onion and garlic plants (Schwartz & Mohan, 2008). Both *F. solani* and *F. oxysporum* (Figure 1.2), for example, are opportunistic fungi that cause damping off in onion that infects germinating seedlings, or the seedlings rot and die before emergence. *Fusarium oxysporum* is also responsible for causing the disease Basal Rot of onion, and Fusarium wilt of watermelons, which infects the stem, and leaf of watermelon (Janick, 2008; Schwartz & Mohan, 2008; Chikh-Rouhou *et al*., 2013).

Fusarium oxysporum is characterised by the production of three types of conidia; microconidia, macroconidia (Figure 1.2) and chlamydospores (Booth, 1971; Leslie & Summerell, 2006). Species delimitation is based on morphological features of the macroconidia however; morphologically identical strains belonging to this species are known to differ regarding pathogenicity (Booth, 1971; Kistler, 1997; Fravel *et al*., 2003; Lori *et al*., 2004; Leslie & Summerell, 2006).

While both pathogenic and non-pathogenic *F. oxysporum* strains are known to exist, many pathogenic strains are host specific. Therefore, the species was subdivided into *formae speciales* and races. To distinguish between pathogenic and non-pathogenic isolates, their ability to cause disease on specific host cultivars must be tested (Correll, 1991; Gordon & Martyn, 1997; Southwood *et al*., 2012a; Southwood *et al*., 2012b). For example, the pathogen *F. oxysporum* f. sp. *cubense* infects banana, *F. oxysporum* f. sp. *lycopersici* infects tomato, and *Fusarium oxysporum* f. sp. *cepae*, infects onion (Lievens *et al*., 2009; Liu *et al*., 2010; Southwood *et al*., 2012a). Also, different *formae speciales* can be identified by classifying strains into vegetative compatibillity groups (VCGs) (Puhalla, 1985; Bayraktar *et al*., 2010).

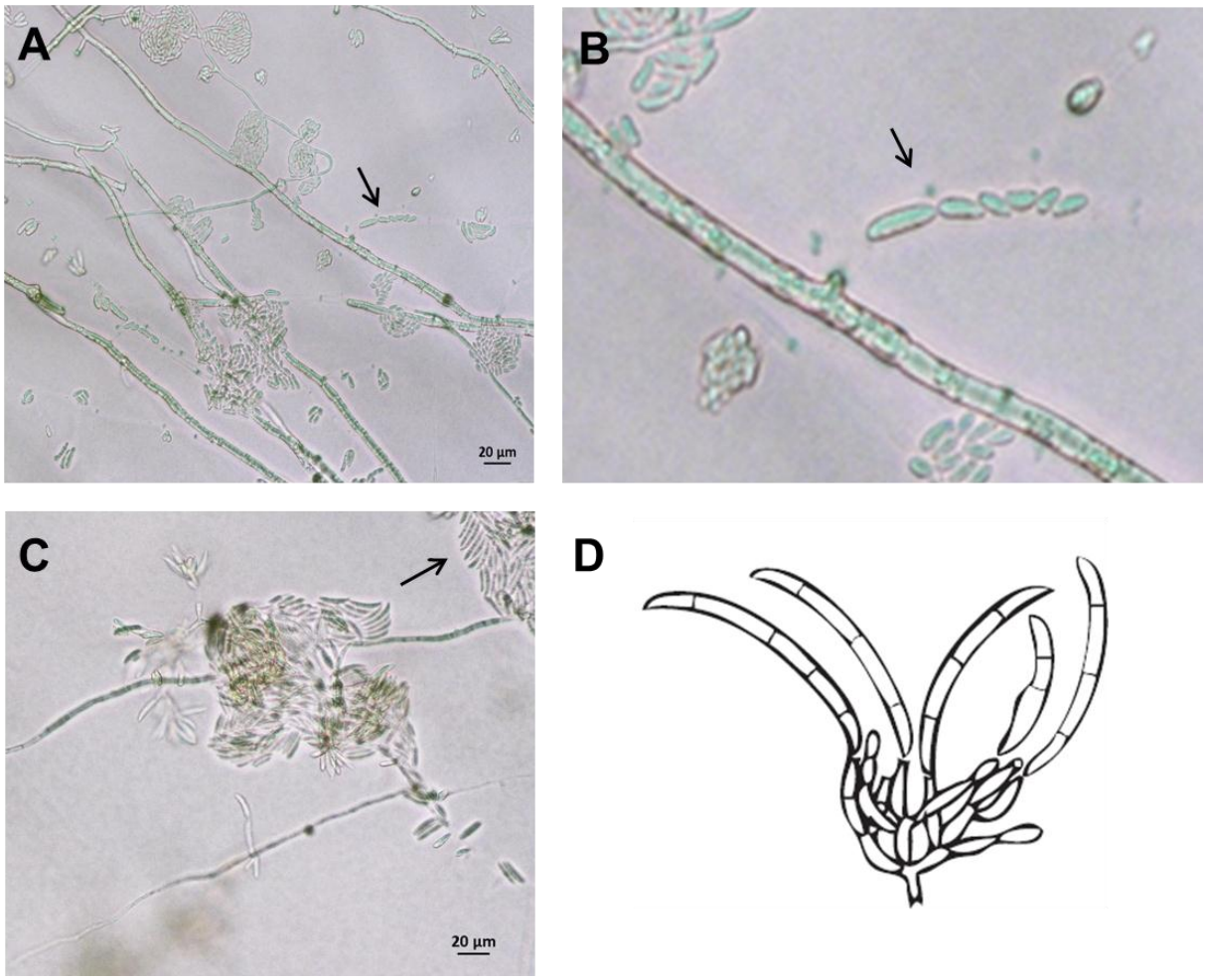

Figure 1.2. Morphology of *Fusarium oxysporum* (CAB 323) grown on CLA (carnation leaf agar) for 14 days at 26°C. The black arrows on the micrographs indicate conidia as visualised using light microscopy. Microconidia are visible in (A), while a magnification of the microconidia is presented in (B). Macroconidia are shown in (C) with a sketch showing characteristic septated macroconidia in (D) (Leslie & Summerell, 2006; picture adapted from Ma, *et al*., 2013).

1.5.2.4.3 Vegetative compatibility groups (VCG)

It has been known for quite some time that insight into the genetic diversity among closely related fungal taxa, especially pathogenic isolates, can be obtained by classification into VCGs (Puhalla, 1985; Bosland, 1987; Leslie, 1996; Galván *et al*., 2008). Thus, physiological complimentation between different mutants of the same species, e.g. *Fusarium oxysporum,* is used to identify the VCG class to which a particular *formae specialis* belongs.

In order for two fungal strains to be vegetatively compatible their hyphae will fuse to form a stable heterokaryon, which will only happen if the isolates are genetically similar (Puhalla, 1985; Leslie 1993; Leslie & Summerell, 2006). The mediater governing VCGs is the vegetative incompatibillity (*vic*) loci. Upon hyphael fusion an initial heterokaryotic cell is formed.

If the *vic* alleles in both the nuclei are identical, the heterokaryon will be stable, if not, the heterokaryon is transitory. The initial heterokaryon will thus be unstable, and is walled off leading to the death of the cell (Arie *et al.,* 2000; Leslie & Summerell, 2006). The strains that form a stable heterokaryon are grouped into a VCG or single-member VCG (SMV; self-compatible isolate which does not anastomose with any other VCG). Strains who do not form stable heterokaryons are deemed vegetatively incompatible and belong to different VCGs. In an asexual population, that is thought to exist for *Fusarium oxysporum,* since sexual recombination has not yet been documented for this species, isolates exhibiting similar pathogenicity traits formed from a clonal lineage are classified into the same VCG.

The classification of *F. oxysporum* isolates using VCG grouping was already employed during the 1980's when it was suggested that a code be assigned to these vegetative compatible isolates (Puhalla, 1985). The first three digits would correspond to the host specialisation (*forma specialis*) with one or two extra digits corresponding to an individual VCG, falling within the *forma specialis*. To determine VCGs, standard techniques are employed as used by Puhalla (1985) and Aloi & Baayen (1993). Nitrate (NO_3^-)-non-utilising (*nit*) mutants are generated to force heterokaryon formation. These mutants form spontaneous sectors on a minimal medium containing $KClO_3$ (potassium chlorate). Sectors are sub-cultured onto minimal medium containing $NaNO_3$ as sole source of nitrogen. If the strains grow thinly on the minimal medium, they are identified as *nit* mutants. The *nit* mutants are classified according to their growth characteristics on a phenotypic medium containing either NH_4^+, NO_3^-, NO_2^- or hypoxanthine as sole nitrogen source. Thus, three types of mutants are recognised based on their ability to assimilate these nitrogen sources, namely, *nit 1, nit 3,* and Nit M.

A *nit 1* mutant cannot grow on medium containing NO_3^- as the sole nitrogen source but will grow on all of the other media. The *nit 3* mutants will not be able to grow on media containing NO_3^- or NO_2^-. When *nit 3* mutants grow with thin mycelia on NO_2^- they are confirmed as *nit 3*. Mutants of the Nit M class can only grow on media containing NO_2^- or NH_4^+ as sole nitrogen source (Leslie & Summerell, 2006). Essentially media containing NH_4^+ are used for positive controls (cultures showing abundant aerial mycelia) while media containing NO_3^- are used as negative controls (cultures showing thin, almost translucent mycelia). Media containing hypoxanthine are used to identify Nit M mutants; the mycelial growth will appear thin, almost translucent. When determining VCGs, pairings between *nit 1* and Nit M are preferred and shows a robust line of mycelial growth on the agar surface representing stable heterokaryon formation. Complementary *nit* mutants of the same strain are paired to see if the strains are heterokaryon self-compatible (HSC) following pairing with *nit* mutants from different strains.

1.6 Using disinfections for microbial control in irrigation water

It was estimated that poor water quality causes approximately 80% of death and illness in the developing world (Schaefer, 2008). However, due to the lack of local fresh water the use of wastewater (poor water quality) for irrigation is widely practiced (Gemmell & Schmidt, 2011). Worryingly, sufficient evidence exists to show the presence of human pathogens on vegetable surfaces irrigated or fertilised with products containing faecal matter. Thus, poor-quality water used for irrigation can cause dangerous contamination of fruit and vegetable crops (Beuchat, 2002; Steele & Odumeru, 2004; Steele *et al*., 2005; Tempelhoff, 2009; Alsanius *et al*., 2010).

1.6.1 Analysing water quality with faecal indicator organisms

Water quality guidelines set by DWAF in South Africa such as the ―Water Quality Guidelines, Volume 4, Agricultural use: Irrigation" published by DWAF in 1996, are used to monitor irrigation water quality. These guidelines have to meet standards set to reduce the numbers of bacteria used as indicators for faecal pollution (Table 1.2). However, these accepted processes may not be sufficient and can even play a meaningful role in the transmission of human pathogens, which cause diseases such as, diarrheal infections and self-limiting gastroenteritis (Heijkal, 1982; Zmirou, 1987; Gerba, 1988; Bosch, 1991; Payment, 1991; Regli, 1991; Mac Kenzie, 1994).

Contamination of irrigation water by means of human pathogens is widely studied (Terzieva & McFeters, 1991; Grabow, 1996; Jamieson, 2002; Steele, 2004; Toze, 2004; Saprykina, 2009; Brassard *et al*., 2012; Jones *et al*., 2014). To test water for all known waterborne pathogens, however, is not feasable. Therefore, biological communities are used to indicate environmental conditions in aquatic ecosystems (Roux *et al*., 1993; Leclerc *et al*., 2001). Faecal coliforms, including *E. coli* are therefore used as indicators of water fitness (DWAF, 1996; Solomon, 2002).

Escherichia coli, found exclusively in all mammalian faeces, cannot multiply outside of a host, and is used as an indicator organism of faecal pollution and should not be present in drinking-water (Edberg *et al*., 2000; WHO, 2008). Nevertheless, *E. coli* is only one of a number of species in the family *Enterobacteriaceae* that are generally referred to as coliforms (WHO, 1996). The so-called thermotolerant coliforms, including *E. coli,* are able to ferment lactose between 44 and 45°C on MacConkey agar, which selects for bile-resistant *Enterobacteriaceae* and differentiates lactose fermenters from non-fermenters of lactose. (Flournoy *et al*., 1990; Atlas, 1993). The presence of these coliforms are used as an indication of faecal pollution by warm blooded-animals. However, some isolates of *Klebsiella, Citrobacter,* and *Enterobacter* also grow and ferment lactose under these conditions, and unlike *Escherichia* are not solely associated with faecal contamination, but are found in vegetation and soils. Nevertheless, the term ‗faecal coliforms' are often used for these bacteria, and the presence thereof is accepted as a standard for assessing water quality (DWAF, 1996; WHO, 1996; Edberg *et al*., 2000).

Microbiological testing for faecal indicator coliforms usually encompasses enumeration thereof as number of colony forming units/100 ml of water (DWAF, 1996). If *E. coli* and faecal coliforms however, are used as an indicator for faecal water contamination, but are not detected in water, it could still contain enteric viruses, protozoa, bacteriophages, and/or bacterial spores, which are more resistant to disinfection (Grabow, 1996).

Table 1.2. The effect of different levels of faecal pollution in irrigation water on crop quality. Adapted from DWAF (1996).

Levels of faecal pollution measured as *E. coli* counts / 100 ml	**Effect on crop quality**
*≤ 1	Little likelihood that human pathogens will spread with application of any irrigation method onto any crop
1 - 1000	Likelihood that human pathogens will be transferred from contaminated vegetables or any other crop eaten raw and of milk from cows grazing on pastures Crops and pastures not consumed raw may be irrigated by any means only after crops and pastures are allowed to dry before harvesting and grazing
> 1000	Provided no contact allowed with humans, water can be used for production of fodder, irrigate tree plantations, nurseries, parks ect.

* Target Water quality range for irrigation water

1.6.2 Water disinfectants for microbial control

One of the main routes via which pathogenic microorganisms can reach produce is through contaminated irrigation water (Jones *et al*., 2014). Irrigation water is typically obtained from groundwater, surface water, or municipal water sources; with surface water sources being considered as the most high risk sources of pathogen contamination. The reason being that these sources are open to many routes by which microorganisms, causing plant infections or human food-borne illnesses, may enter.

To control these waterborne diseases water sources are physically and/or chemically treated to remove pathogenic organisms. This results in a water source suitable for agricultural application or human consumption (Binnie *et al*., 2002).

Several conventional methods are used to remove human and animal pathogens from irrigation water. For partial removal thereof; coagulation, sedimentation, absorption and flocculation is used (physical treatment). These treatments however, do not fully inactivate microorganisms, therefore water is also chemically treated during or after the physical treatment stage (DWAF, 1996). After water is disinfected a residual treatment is applied so that water remains safe after leaving a water treatment facility (Mahajan *et al*., 2009). Popular treatments for removal of potential pathogenic microorganisms include chlorine addition, as well as treatment with ozone and ultraviolet (UV) light (WHO, 2008; Jones *et al*., 2014).

Ozone is the most energetic process (chlorine being lesser) and is described as most active against microorganisms. Bacteria and viruses are rapidly killed, with parasite cysts significantly reduced in viability. This process, unlike chorine addition, does not leave a residual that provides protection against post-treatment contamination, but does produce bromate as a by-product (WHO, 2008; Edberg *et al*., 2000).

Ultraviolet (UV) light technology can also be applied to inactivate microorganisms (WHO, 2008). This technology is non-chemical and utilises low-pressure mercury arc lamps. The lamps produce germicidal monochromatic UV radiation at a wavelength of 254 nm. Water in vessels or flow-through reactors is exposed to the UV radiation from these lamps at sufficient doses for inactivating waterborne pathogens. In a recent study by Jones and collaborators (2014), UV radiation was effective in removing 99.9% of pathogenic oomycetes and bacteria from surface water used to irrigate fruit and vegetable crops. This technology, however, is not cost-effective because of the need for an electrical supply and high maintenance costs. To date the most common and cost-effective disinfectant for water treatment remains to be calcium hypochlorite (Tully, 1914; WHO, 2008; Migliaccio, 2009; Garcia-Villanova *et al*., 2010; WHO, 2013).

1.6.2.2 Disinfection with Chlorine

During the 1890‘s, sanitary engineers found that by treating drinking water with chlorine, the water was rendered free of pathogens (Edberg *et al*., 2000). This was an inexpensive, effective, and very simple method. The use of disinfection, to produce pathogen-free water, thus began. The primary purpose of wastewater chlorination is to destroy or deactivate microorganisms that cause diseases. In addition, it was found that chlorine treatment improves the overall water quality by reacting with ammonia, manganese, iron, sulphide, and some organic substances. However, adverse effects may exist since the reaction of chlorine with phenols and other organic compounds may result in the taste and odour characteristics of these organic compounds to intensify (APHA *et al*., 2012).

Chlorine is a non-selective oxidiser; reacting with a variety of cellular components of microorganisms, effecting metabolic processes (Shang, 1999). Classified as a low-level disinfectant, chlorine kills most vegetative bacteria, some fungi and certain viruses within a given contact time (Virto, 2004). However, this disinfectant does not work effectively against protozoan pathogens, particularly *Cryptosporidium* (WHO, 2008).

For chlorination to work effectively on irrigation water, proper injection methods, and appropriate concentration of chlorine, must be used to prevent damage to irrigation systems and to prevent harming of agricultural crops (Migliaccio, 2009). Solid chlorine for example, is not recommended for irrigation purposes. It has been stated that when calcium hypochlorite is used on a clogged irrigation system (caused by microbial growth) the calcium may react with other elements in the water, which in turn may cause precipitates to form and clog micro-irrigation emitters. Thus, when an irrigation water source is high in minerals, liquid chlorine is recommended for chlorination (Migliaccio, 2009). Nevertheless, the most common chlorine disinfectant remains to be calcium hypochlorite, which is used to chlorinate a wide range of different water sources (Tully, 1914; Migliaccio, 2009; Garcia-Villanova *et al*., 2010; WHO, 2013). Generally, calcium hypochlorite contains 65% to 70% of available chlorine. Hypochlorous acid (HOCl) and hydroxyl ions (OH) form after hypochlorite acid dissolves in water raising the water pH. According to WHO in order for chlorine to be most effective against microorganisms, chlorine must be added to water and have a contact time for 30 min at a temperature of 18 C or above. If the water temperature is lower then the contact time should be increased. In addition, chlorine was found to be most effective under acidic conditions (WHO, 2005; WHO, 2008; Migliaccio, 2009). When chlorine destroys an organism there is a subsequent loss of available chlorine (WHO, 2005). However, when high concentrations of chlorine are used as a disinfectant, some available chlorine may remain in the water. This is called free chlorine (also residual chlorine) and may be lost at a later stage to the atmosphere or utilised when destroying new contaminants. The free chlorine can be tested for by using methods described in Standard Methods (APHA *et al*., 2012). When high levels of residual chlorine are measured, it may indicate that enough chlorine was initially added to the water to destroy most pathogens. The levels of residual chlorine regarded as acceptable range between 0.50 and 0.20 mg/L. When residual chlorine concentrations are below 0.20 mg/L, more chlorine must be added to the water (WHO, 2005).

1.6.2.2.1 The effect of turbidity on chlorine disinfection

Impediments which may hinder chlorine disinfection include turbidity. For chlorine to be most effective, it must be in direct contact with the organisms; meaning water needs to be clear of silt or sand, i.e. it must have a low turbidity (WHO, 2008; WHO, 2011).

Turbidity is the result of sediments or inorganic particulate matter present in source water and can be caused by silt, sand, mud, as well as bacteria or chemical precipitates; and is measured in nephelometric turbidity units (NTU) or Jackson turbidity units (JTU). The two units are roughly equal (WHO, 2008).

Turbidity may deter disinfection by protecting pathogens within flocs or particles where the disinfectant cannot penetrate (WHO, 2008; WHO, 2011). These particles thus reduce the efficacy of the particular disinfectant, resulting in a higher demand for chlorine to effectively disinfect water. So far, no health-based guideline value has been proposed but it is known that turbidity needs to be below 1 NTU for effective disinfection; however for drinking purposes, the appearance of water with a turbitity less than 5 NTU, is usually acceptable to consumers.

1.7 Aim of study

The aim of this study was firstly to screen irrigation and surface water of the Lower Vaal River for faecal contamination by using standard methods set by DWAF (1996), i.e. utilising MacConkey agar for the enumeration of faecal coliforms (Chapter 2). Secondly, the study aimed to determine whether water from the Lower Vaal River used for irrigation purposes, contained species of the waterborne plant pathogen *F. oxysporum,* capable of infecting onion bulbs cultivated on nearby land (Chapter 3). *Fusarium* isolates, obtained from both Lower Vaal River samples and onions collected from nearby fields were tested for pathogenicity on onion bulbs. Pathogenic *F. oxysporum* isolates were subsequently classified using VCG grouping. Finally, the efficacy of the cost effective calcium hypochlorite containing disinfectant, ‗HTH® Super Shock It', to remove potential plant pathogenic *Fusarium* spores and faecal coliform bacteria, was determined in samples of river water, differing in pH, turbidity, and temperature (Chapter 4).

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Chapter 2

Detecting faecal contamination in Lower Vaal River irrigation water

2.1 Introduction

The Vaal River arises on the High Veld plateau of Mpumalanga and eastern Free State (South Africa). Its 1,350 km course, mostly elevated between 1,400 and 1,800 m above sea level, was primarily formed by shales and sandstones of the Karoo system (Butzer, 1973). The end of the Vaal River's course in the Northern Cape, it flows west past Riverton and Willowbank, and finally joins the Orange River at 957 m above sea level near Douglas.

As the most significantly utilised river in the country, the quality of water sources of the Vaal River are immensely important (DWAF, 2009; DWAF, 2011). Consequently, the Vaal River system was subdivided into three water management areas (WMA; Chapter 1). Serving as a conduit the Vaal River transfers water between these three WMAs. The main source of water for the Lower Vaal WMA, where the focus of the present study lies, is the surface flow from the Upper and Middle Vaal WMAs. The Lower Vaal WMA is subjected to extensive livestock farming, crop production and dry land cultivation (DWAF, 2009). Not surprisingly, approximately 80% of the water in the Lower Vaal WMA is used for irrigation practices (DWAF, 2009). Worryingly, it was stated that the water quality of the Lower Vaal River has been deteriorating over the past 20 years (Du Preez *et al*., 2000). It was also contented that the situation is not likely to improve, since a large portion of the water ending up in the Lower Vaal WMA is wastewater originating from Gauteng, the most densely populated province in South Africa.

It is well known that population growth coupled with global climate change and extreme weather events exert strain on natural resources, such as water (Department of Economic and Social Affairs, 2011; Rothamsted Research, 2012). In addition, South Africa has no surplus water to dilute out harmful pollutants, originating from wastewater, in water sources such as river systems (Turton, 2008). These harmful contaminants may end up in irrigation water and may include both chemical and biological pollutants (DWAF, 1996). Biological pollutants include human and animal pathogens and parasites that infect, and survive, on field crops and vegetables. It is therefore of the utmost importance to ensure that the quality of irrigation water used in agriculture, complies with guidelines set by authorities.

Quality guidelines for irrigation water in South Africa are set by the DWAF (Department of Water Affairs and Forestry) (Chapter 1; DWAF, 1996). These guidelines are also in place to detect harmful human gastrointestinal pathogenic bacteria, such as *Escherichia coli* 0157:H7, previously found in the tissue of lettuce plants that were irrigated with manure contaminated water (Solomon, 2002). Other studies also revealed the dangers of irrigating crops with faecal contaminated water, showing that *E. coli* 0157:H7 can survive for longer periods of time in these waters than its benign counterparts (Erickson *et al.*, 2010; Fonseca *et al.,* 2011; Mauro *et al.*, 2013), thus highlighting the need for higher quality irrigation water.

It is common practice to determine the biological quality of water, including that of irrigation water, by enumeration of cultureable faecal coliforms, including *E. coli* (DWAF, 1996; Steele & Odumeru, 2004; Steele, *et al.,* 2005). Recently however, a rapid detection system, the 3M™ Molecular Detection System (3M™, Minnesota, U.S.A), was developed to reveal pathogenic faecal coliforms in food products. This system was initially designed to identify *E. coli* 0157:H7, *Listeria* spp. (species) and *Salmonella* spp within 75 min (3M Food Safety/3M Molecular Detection system brochure, 2014). Recently, the system was also successfully used to detect these harmful human pathogens in wastewater samples (Loff *et al*., 2014).

With the above as background, the aim of this study was to detect culturable faecal coliforms in water from the Vaal River using dilution plates prepared with MaConkey agar (APHA *et al*., 2012). In addition, the 3M™ Molecular Detection System was used to determine whether *E. coli* 0157:H7, *Listeria* spp. or *Salmonella* spp. were present in water from the Vaal River.

2.2 Materials and Methods

2.2.1 Sampling site and procedure

The surface water samples were collected at 15 undisclosed sampling sites situated in five sampling areas along a 159 km stretch of the Vaal River, in the Northern Cape (Figure 2.1; Table 2.1). In total 63 water samples were collected at random over a period of three years (09 May 2011 until 05 June 2013). Sterile 5 L plastic bottles were used for sampling and the samples were analysed within 24 hours after collecting the water.

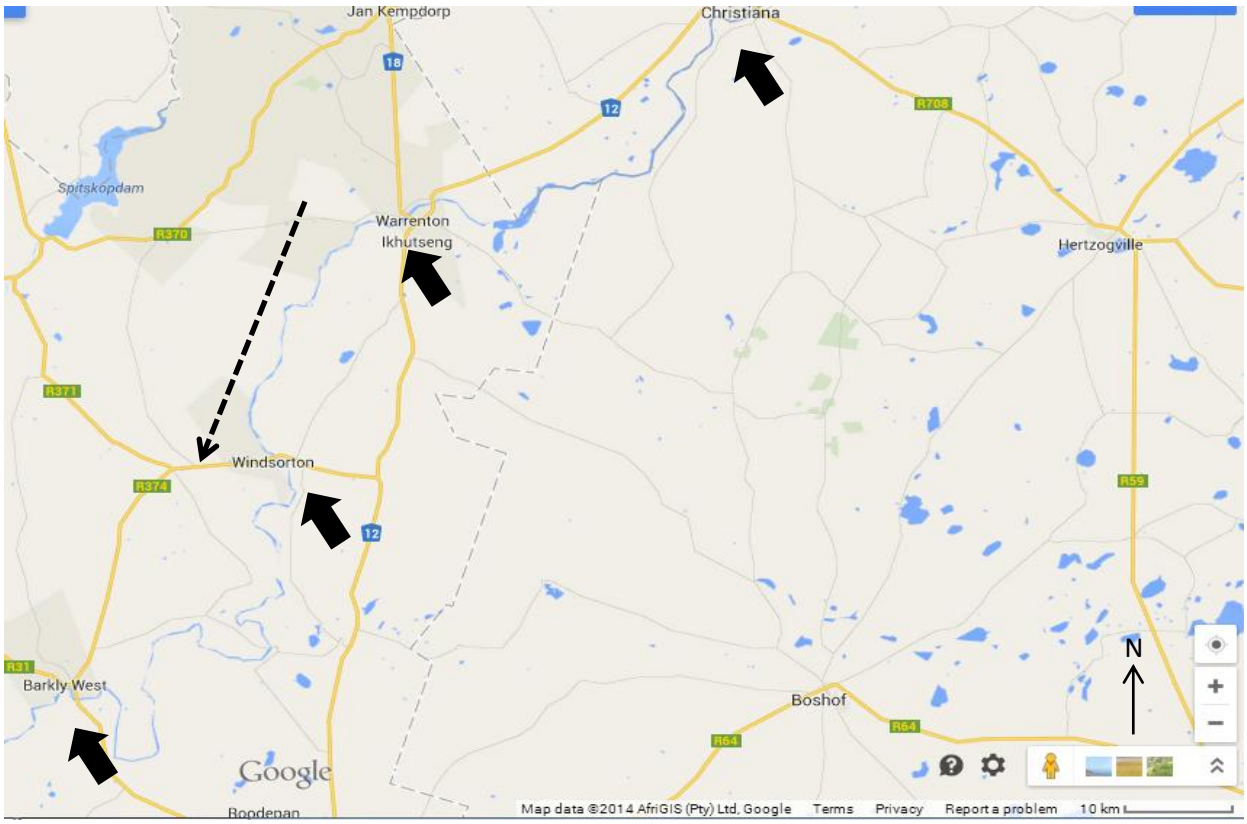

Figure 2.1. Map of a region where the water samples were collected along the banks of the Vaal River. The map shows (solid arrows) the location of four of the five different areas (excluding Bloemhof), i.e. Barkley West, Windsorton, Warrenton, and Christiana. Dotted arrow indicates flow of river (Google maps).

Table 2.1. River water samples were collected at different sites, either directly from the river or from a pump station in five areas along the banks of the Vaal River.

Date	Area	Sampling Site	No. of samples collected
09 May 2011	Barkley West	S1	Triplicate
09 May 2011	Barkley West	S2	Triplicate
31 May 2011	Barkley West	S2	Triplicate
26 Aug 2011	Barkley West	S2P1	Single
		S2P2	Single
		S2P4	Single
		S2P3	Single
		S2P5	Single
	Warrenton	S1	Single
	Bloemhof	S1	Single
	Windsorton	S1	Single
	Christiana	S1	Single
30 Nov 2011	Barkley West	S2P3	Single
		S2	Duplicate
09 Sep 2012	Barkley West	S2	Triplicate
		S1P3	Triplicate
		S1	Triplicate
08 Dec 2012	Barkley West	S1	Triplicate
		S1	Triplicate
01 Feb 2013	Barkley West	S1	Triplicate
		S3	Triplicate
		S4	Triplicate
20 Feb 2013	Barkley West	S1	Triplicate
		S3	Triplicate
5 June 2013	Barkley West	S1	Triplicate
		S3	Triplicate
		S4	Triplicate
		S5	Triplicate

"S" denotes sampling site, while "P" denotes pump station.

2.2.2 Culturing and enumeration of faecal coliforms

To enumerate faecal coliforms, the water samples were serially diluted and spread onto MacConkey agar (Merck, Darmstadt, Germany) in triplicate, where after the plates were incubated for 24 h at 44°C. Faecal coliforms were identified and recorded as colonies with a red or pink hue (Atlas, 1993). The numbers of dark red colonies, showing a halo of precipitated bile, were also enumerated since these are considered to be representing *E. coli* (APHA *et al*., 2012).

2.2.3 Detection of *E. coli* 0157:H7, *Listeria* and *Salmonella* using 3M™ Molecular Detection System

The 3M™ Molecular Detection System was supplied by 3M™ South Africa and was used to test for the presence of *E. coli* 0157:H7, *Listeria* and *Salmonella* in the river water samples, according to the manufacturer's instructions. The system identifies these pathogens by using isothermal DNA amplification and bioluminescence (3M Food Safety/3M Molecular Detection system brochure, 2014). This detection system allows for the DNA amplification of a particular organism in real time with negative results indicated at the end of the run. Each run has a duration time of 75 min with positive results indicated after 15 min.

Enrichment of *Salmonella* spp. and *E. coli* 0157:H7 was achieved by adding 12.5 ml of the water sample to 225 ml pre-warmed 3M™ buffered peptone water (BPW-ISO) and for the enrichment of *Listeria* species, 12.5 mL of the river water sample was added to Modified *Listeria* recovery broth (3M™, South Africa). The enrichment media were subsequently incubated for 24 h at 37°C (± 1°C). Following incubation, 20 µl of each enriched sample broth was transferred to a lysis tube and incubated for 15 min at 100°C (± 1°C). The lysis tubes were then transferred to a cooling block for 10 min, and incubated for 5 min at room temperature (ca. 20°C). The lysate (20 µl) of each sample was then transferred into colour coded reagent tubes, containing lyophilised pellets. Tubes were transferred to a speed loader tray which was placed into the 3M™ Molecular Detection System and the sample run was activated. Positive and negative controls were added (discussed in later section).

2.3 Results and Discussions

2.3.1 Culturing and enumeration of faecal coliforms

South African water quality guidelines for agricultural use indicate that the presence of faecal coliforms may be viewed as an indication of the presence of bacterial pathogens (DWAF, 1996). These include representatives of *Salmonella*, *Shigella*, *Vibrio cholerae, Campylobacter jejuni* and *E. coli*.

When crops, irrigated with contaminated water are consumed raw these organisms may be transmitted via the faecal/oral route and may cause enteric diseases. These diseases include, typhoid fever, gastroenteritis, dysentery, salmonellosis and cholera (DWAF, 1996). It was thus imperative that the amount of faecal coliforms in irrigation water be within the target quality range set by DWAF. The Department of Water Affairs and Forestry have indicated that if the numbers of faecal coliforms (*E. coli*) are between 1 - 1000 CFU/100 ml, this water will contaminate produce consumed raw, resulting in the spread of human pathogens. As indicated in Table 2.2, all the samples collected contained notable levels of faecal coliforms, increasing the risk of produce contamination by *E. coli*. These high levels of faecal contamination in the Vaal River were observed throughout the entire sampling period. Along the banks of the Lower Vaal River livestock farming takes place and urban settlements are present (DWAF, 2004c). These factors may have contributed to faecal contamination of the river.

Table 2.2. Indications of the average faecal coliforms and *E. coli* per 100 ml of irrigation water. Samples were randomly sourced along the banks of the Vaal River including various pump stations, over a period of three years. Faecal coliforms were enumerated on MaConkey agar after incubation for 24 h at 44°C.

Area	Sampling Site	Average faecal coliforms detected (CFU/100 ml)	Average *E. coli* detected (CFU/100 ml)
Barkley West	S1	3.85×10^4	nd
Barkley West	S2	7.22×10^4	nd
Barkley West	S2	9.77×10^4	nd
Barkley West	S2P1	8.70×10^3	nd
	S2P2	4.60×10^5	nd
	S2P4	2.00×10^3	nd
	S2P3	2.70×10^4	nd
	S2P5	1.37×10^4	nd
Warrenton	S1	2.62×10^5	nd
Bloemhof	S1	2.80×10^5	nd
Windsorton	S1	7.40×10^4	nd
Christiana	S1	1.85×10^5	nd
Barkley West	S2P3	5.70×10^3	nd
	S2	2.14×10^6	nd
	S2	4.03×10^4	2.23×10^4
Barkley West	S1P3	4.73×10^6	≤1
	S1	2.87×10^6	≤1
Barkley West	S1	1.61×10^5	≤1
	S1	7.80×10^5	≤1
Barkley West	S1	2.74×10^5	≤1
	S3	3.79×10^5	≤1
	S4	5.79×10^5	4.33×10^2
Barkley West	S1	6.67×10^4	≤1
	S3	1.27×10^5	≤1
Barkley West	S1	1.70×10^4	3.33×10^2
	S3	1.13×10^4	1.00×10^3
	S4	1.83×10^4	≤1
	S5	5.00×10^3	3.33×10^2

nd, denotes not determined; ≤, denotes lower than the detection limit

2.3.2 Detection of *E. coli* 0157:H7, *Listeria* and *Salmonella* using 3M™ Molecular Detection System

An example of an organisation graph obtained after a sampling run is shown in Figure 2.2. Reagent tubes transferred into the speed loader tray of the 3M™ Molecular Detection System, are colour coded in order for differentiation between assays, i.e., green for the detection of *Salmonella* spp, (lane 1), pink for *E. coli* 0157:H7 (lane 2) and blue for *Listeria* spp. (lane 3). The negative controls and reagent controls (representing positive controls) were loaded in lanes B and C (Figure 2.2). Matrix controls for both BPW-ISO broth (detection of *Salmonella* spp.) and *E. coli* 0157:H7, as well as 3M™ Modified *Listeria* recovery broth (detection of *Listeria* spp.), were loaded in lane D. The matrix control is required in order to detect and report on sample inhibition that may occur during the amplification step of the system (3M Food Safety/3M Molecular Detection system brochure, 2014). Enriched Vaal River water samples were loaded into lane A (Figure 2.2). Results from the detection assay showed no indication of *Salmonella* or *Listeria* (Figure. 2.2 lane A1 and A3) as revealed by the negative symbol in green. However, *E. coli* 0157:H7 was detected in the water using the Molecular Detection System as indicated by the positive symbol in red (Figure 2.2 lane A2). This may be because these organisms were not viable or do not survive very long in the river water after sampling (Mauro *et al*., 2013). The matrix control is visible as a blue colour, indicating that no inhibition occurred during the detection assay (Figure 2.2, lane D). From Figure 2.2, it is also apparent that the negative controls and reagent controls are valid after the sample run, as indicated in blue. The occurrence of *E. coli* 0157:H7 has been discovered before in the Vaal River Barrage as reported by Muller (2001), although infrequently detected. The author concluded that water, which may contain some of the human pathogen's virulence factors, could potentially be a health risk if ingested, further proven in a study conducted by Mauro and co-workers (2013).

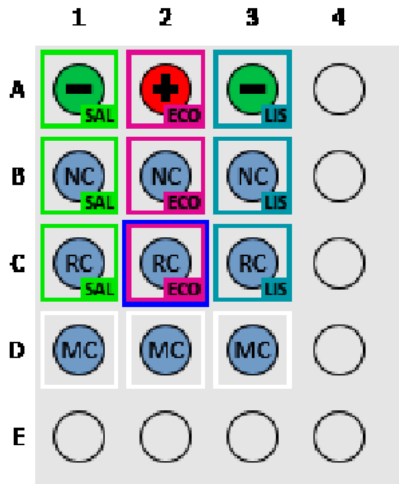

Figure 2.2. Section of a run report for enriched Vaal River water samples using the 3M™ Molecular Detection System. Where NC = negative control, RC = reaction control (positive control) and MC = matrix control. Positive results are shown as red (+) with negative results shown in green (-). Valid results for RC, NC and MC are shown in blue.

2.4 Conclusions

Results from this detection study reveal that the Vaal River is contaminated with faecal organisms, which may cause various enteric diseases. In addition, the 3M™ Molecular Detection System confirmed the presence of the human pathogen *E. coli* 0157:H7 in the river.

It thus seems that the Vaal River is heavily polluted as a result of anthropogenic activities along its banks. It is strongly advised that disinfection processes be initiated to combat these potential pathogens in irrigation systems. However, it is not only human pathogens that are of concern, but plant pathogens may also occur in irrigation water subjected to anthropogenic activities (Bucheli *et al.,* 2008; Summerell *et al.*, 2010; Van Wyk *et al*., 2012).

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Chapter 3

Isolating and characterising *Fusarium oxysporum* isolates associated with onion production along the Lower Vaal River

3.1 Introduction

South Africa's agricultural sector, worth R60 billion in total gross domestic product, is considered an important component of the country's economy. Agriculture provides employment in rural areas and is a significant stipendiary of foreign exchange (Department of Agriculture, 2011).

Coupled with a growing world population, currently at seven billion, estimated to surpass nine billion by 2050, demands for global food production will increase (OECD-FAO, 2009; Department of Economic and Social Affairs, 2011). However, one of the challenges facing sustainable food production is plant and human pathogens that reduce food quality and safety. These pathogens may contaminate food when introduced to crops during irrigation with polluted water (Steele & Odumeru, 2004; Steele *et al.,* 2005). Worryingly, polluted river water is often used for irrigation in developing countries, including South Africa. The Vaal River in South Africa for instance, a major water source used in agriculture and industry, is known to be polluted. Affected by sewage pollution, expanding urban areas, and extensive industrial and mining developments, most pollutants originate from, but are not limited to, the Upper and Middle Vaal water management areas (WMA) which end up in the Lower Vaal WMA (DWAF, 2009; Tempelhoff, 2009). In addition, agricultural surface runoff ending up in rivers may contain waterborne plant pathogens which may pose a threat to production when introduced to crops via irrigation (Bucheli *et al.,* 2008; Summerell *et al.*, 2010; Van Wyk *et al*., 2012). It is thus tempting to speculate that irrigation water from the Lower Vaal WMA, where large-scale onion cultivation takes place, harbour waterborne plant pathogens that may infect crops.

Onion (*Allium cepae* L.), considered one of the world's most important fresh vegetables, comprises 21.9% of the agricultural land planted vegetables in the world. In South Africa, onion is also an important vegetable crop, with 7000 ha cultivated on an annual basis (Southwood, 2010). However, onion production is prone to attack by plant pathogens. One of these pathogens is *Fusarium oxysporum* f. sp. *cepae* (*Focep*) W. C. Snyder & H. N. Hansen (1945), which causes Fusarium basal rot, a disease of worldwide importance causing both field and storage losses. Symptoms consist of a brownish, watery rot within infected onions, (Abawi & Lorbeer, 1971; Schwartz & Mohan, 2008; Southwood *et al*., 2012b) making them undesirable for human consumption. The first report of Fusarium basal rot in the Western Cape by *Focep* was by Doidge *et al.* (1954). Following this report, Holz in 1973 recorded serious losses of harvested onion by the causal agent *Focep* on certain farms in Caledon-Riviersonderend district within the Western Cape (Holz & Knox-Davies, 1976). Interestingly, although intensive onion production in the Northern Cape has a long history, major losses from basal rot, as a result of *F. oxysporum,* were only noted recently (personal communication with farmer; Southwood *et al*., 2012b). In South Africa, regions within the Western Cape (Koue Bokkeveld) now suffer severe losses due to basal rot.

The disease is less prevalant in other regions such as Limpopo, Boland, and the southern and northern Cape (Southwood, 2010; Southwood *et al*., 2012b). *Focep* forms part of the *F. oxysporum* species complex that includes more than 120 different *formae speciales* that are morphologically indistinguishable. These *formae speciales* are further subdivided into races, based on their reaction on a set of differential host cultivars (Correll, 1991; Aloi & Baayen, 1993), and vegetative compatibillity groups (VCG). VCGs are governed by vegetative incompatibillity (*vic*) loci. When two fungal isolates are genetically similar, their hyphae will fuse to form a stable heterokaryon. This stabillity implies that the two strains belong to the same VCG (Puhalla, 1985; Leslie, 1993). A single mutation at the vegetative incompatibillity locus averts the formation of a stable heterokaryon, causing closely related isolates to belong to different VCGs (Leslie, 1993). Isolates with similar pathogenic capabilities (races) include different VCGs, while the same VCG can also belong to different races (Leslie & Summerell, 2006).

Among *Focep* isolates, seven known VCGs and several single-member VCGs (SMV) have been identified in South Africa. These include VCGs 0420-0426 and SMVs 1-3 (Yoo *et al*., 1993; Swift *et al*., 2002; Southwood, 2010). VCG 0425, found to be the predominant *Focep* VCG in South Africa was found to be highly virulent while VCG 0423; present in South Africa, Turkey and Colorado; appears to be less virulent to onions (Bayraktar *et al*., 2010; Southwood, 2010).

Pathogenicity testing is required to identify *formae speciales* of *F. oxysporum*, except in cases where molecular markers were developed (Lievens *et al.,* 2008). To determine pathogenicity of *F. oxysporum* isolates to onion, a basal plate inoculation method was developed by Southwood (2010). Molecular identification systems that have been developed for *formae speciales,* include amplified fragment length polymorphysm (AFLP) analysis (Baayen *et al*., 2000; Vakalounakis *et al.,* 2005) and random amplified polymorphic DNA (RAPD) analysis (Clark *et al.,* 1998; Haan, 2000; del Mar Jiménez-Gasco *et al.,* 2001; Vakalounakis *et al.,* 2004). In addition, sequence characterised amplified region (SCAR) markers have been developed (Southwood *et al.,* 2012a). More recently, 'secreted in xylem' (*SIX*) genes were found to be the virulence factors associated with the tomato fungal pathogen *F. oxysporum* f. sp *lycopersici* (*Fol*), the casual agent of Fusarium wilt of tomato (Lievens *et al.,* 2009). However, these genes were not limited to *Fol,* and have since been found in other *formae speciales* such as *F. oxysporum* f. sp. *lilii*, *F. oxysporum* f. sp. *melanis* and *F. oxysporum* f. sp. *cubense*, making them important markers for *F. oxysporum* characterisation (Lievens *et al*., 2009; Meldrum *et al*., 2012).

With the above as background the aim of this study was to determine whether irrigation water from the Vaal River, and nearby onion plantations, contained the waterborne plant pathogen *Focep.* The pathogenicity and VCG identity of *F. oxysporum* isolates collected from the river and onion fields were also determined. Finally, all *F. oxysorum* isolates were screened for the presence of *SIX* genes.

3.2 Materials and Methods

3.2.1 *Fusarium* isolations from Vaal River and onion bulbs

Sampling was conducted in the Northern Cape Province in onion fields and from irrigation water along the Vaal River between May 2011 and November 2013 (Chapter 2; Table 3.1). Irrigation water samples were collected in sterile 5 L bottles directly from the river or at pump stations leading towards centre-pivot irrigation systems, and delivered to the Department of Microbiology, Stellenbosch University on ice. Sub-samples (500 ml) were subsequently filtered through filter paper (Munktell grade 3, 125 mm, Lasec, SA) using a Buchner funnel (125 mm, Lasec, SA) under vacuum.

The filtrate was aseptically transferred to sterile glass bottles containing 9 ml distilled water. One ml of the resulting suspension was spread plated onto replicate plates prepared from potato dextrose agar (PDA; Merck, Darmstadt, Germany) supplemented with 0.50 g/L streptomycin sulphate and 0.20 g/L chloramphenicol (PDA*). In addition, onion bulbs, symptomatic and asymptomatic, were collected on 30 November 2011 and 03 December 2012 and tested for *Fusarium* infection. Isolations were made from one symptomatic and seven asymptomatic onion bulbs (Lombardi cultivar) by aseptically cutting the bulbs in half. Small tissue sections of 1 to 3 mm in diameter, dissected from inside the basal plate, were then placed on PDA* plates.

All plates were incubated at 26°C, and inspected regularly for fungal growth. Within 3 to 5 days, *Fusarium* spp. were collected by single-spore isolation. *Fusarium* isolates were tentatively identified to species level by means of conidial morphology as described by Leslie and Summerell (2006). All isolates obtained during this study were stored in 30% glycerol at -80°C and can be found in the culture collection at the Department of Microbiology under the reference CAB.

3.2.2 Identification of *Fusarium* isolates

3.2.2.1 Genomic DNA extraction

Mycelia from 2-week-old *Fusarium* cultures were harvested from PDA plates by scraping off mycelia using a sterile surgical blade. DNA was extracted using a ZR Fungal/Bacterial DNA MiniPrep™ system (Zymo Research, California, USA) according to the manufacturer's recommendations.

3.2.2.2 Confirmation of species identity

The identity of the *Fusarium* spp. tentatively classified based on their morphology were confirmed by sequencing of the translation elongation factor 1-α (EF) gene. Primers EF-1 (5'-ATGGGTAAGGAAGACAAGAC) and EF-2 (5'- GGAGGTACCAGTCATCATGGT) (O'Donnell *et al*., 2004) were used in the PCR amplification of the EF-1 α gene. The PCR reaction volume consisted

of 25 µl PCR master mix (2X) (Thermo Scientific, South Africa), 1 µl (10 µM) of each primer and 5 µl genomic DNA in a final reaction volume of 50 µl. An Applied Biosystems 2027 Thermal cycler was used for amplification, using an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 7 min. Agarose gel (0.8%) stained with ethidium bromide was used to separate amplicons. PCR products were purified by treatment with SDS (sodium dodecyl sulphate) and transferred onto Sephadex columns. These columns were then transferred to a Tecan EVO150 following centrifugation. Cleaned sequencing reaction products were dried using a heated vacuum drier then re-suspended in Hi-Di (Applied Biosystems). Sequencing reactions of PCR products were conducted in both directions using the BigDye Terminator sequencing kit (Applied Biosystems) in accordance to the manufacturer's specifications. Geneious Pro 3.5.6 (Biomatters Ltd., Auckland, New Zealand) was used to view sequencing files to obtain consensus double strand sequences for every isolate. The sequences were subsequently subjected to nucleotide BLAST analysis in the FUSARIUM-ID database v 1.0 (http://isolate.fusariumdb.org) (O'Donnell *et al*., 2010).

3.2.3 Pathogenicity testing

The *Fusarium* isolates (Indicated by CAB numbers; n = 57) collected from onion fields and Vaal river water were subjected to pathogenicity testing using the basal plate inoculation method developed by Southwood (2010) (Table 3.2). Bulbs of *Allium cepa L.* cultivar Lombardi, cultivated in the Northern Cape Province, was used for pathogenicity tests. This cultivar is economically important and highly susceptable to Fusarium basal rot. Inoculum was prepared by inoculating hyphae of the *Fusarium* isolates into 250 ml Erlenmeyer flasks containing a 100 ml of Armstrong medium (Booth, 1971). These flasks were placed on a rotary shaker at 125 rpm and rotated for 10 days at 26°C. After incubation, spores were filtered through two layers of muslin cloth to remove all the mycelia (Booth, 1971). For each isolate, a spore concentration of 5 x 10^4 spores/ml was used to inoculate basal plates of five wounded onion bulbs per isolate.

Before inoculation, the dry scales and one layer of inner flesh scale, as well as the roots of onion bulbs were removed. The basal plates of onion bulbs were then wounded with a sterile scalpel (20 shallow, 5 mm wounds per plate), followed by inoculation with a 1 ml spore suspension.

Bulbs were dried in a laminar flow cabinet and then incubated in sterile brown paper bags in moisture chambers for 6 weeks (Figure 3.1). The moisture chambers consisted of clear Perspex cases of 30 x 60 cm, with a sheet of chromatography paper lined inside the base, resting in a metal tray containing deionised water. Incubation took place at 24 to 26°C, with 60% relative humidity and natural light conditions. After 6 weeks of incubation, the onions were cut in half and evaluated for infection.

Pathogenic *Fusarium* isolates were re-isolated onto PDA* and tentatively identified as *F. oxysporum* using conidial morphology to fulfill Koch's postulates following conformation of species identity as described in section **3.2.2.2**. The negative control used in this study was sterile distilled water. The positive control was a highly virulent *Focep* isolate, STE-U 6643, previously characterised by Southwood (2010) and assigned to VCG 0425.

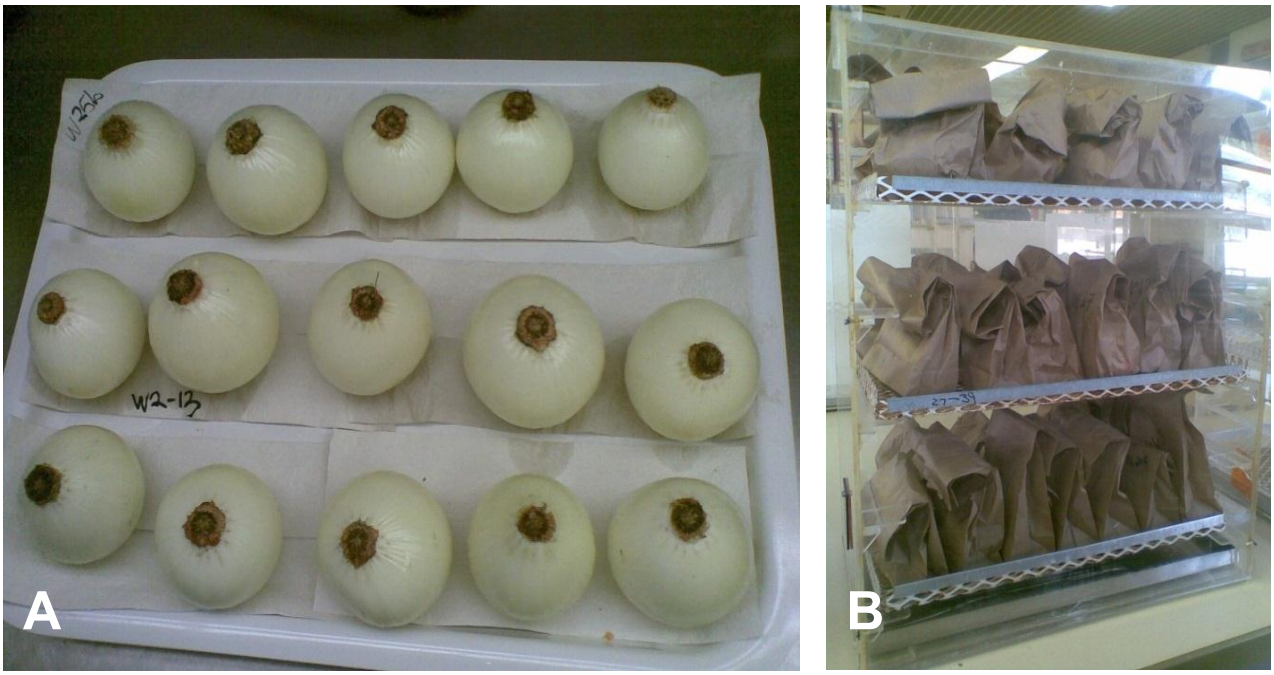

Figure 3.1. Onions infected with *Fusarium oxysporum* f. sp. *cepae* and left to dry in laminar flow (A). Each onion was packed in a sterile paper bag and incubated in a disinfected moisture chamber for 6 weeks (B) under natural light condition.

3.2.3.1 Classification of pathogenic isolates according to vegetative compatibility groups

The VCG status of all *F. oxysporum* isolates pathogenic to onion (Southwood, 2010), was determined using standard techniques (Puhalla, 1985; Aloi & Baayen, 1993). Nitrate non-utilising (*nit*) mutants were generated on a solid basal medium containing 4% potassium chlorate ($KClO_3$) (Aloi & Baayen, 1993), and transferred to minimal medium (MM) slants. Only the colonies that grew as thin, non-aerial mycelium on MM were deemed *nit* mutants, indicating a loss in the isolates ability to utilise nitrogen present in the medium (Puhalla, 1985).

For isolates which failed to create *nit* mutants the concentration of chlorate was altered or the addition of $NaNO_3$ was omitted. The *nit* mutants were thereafter classified into *nit 1, nit 3,* and Nit M mutants based on their growth on a phenotypic medium consisting of MM and one of the following nitrogen sources: NH_4^+, NO_3^-, NO_2^- and hypoxantine.

VCG identity of the *Fusarium* isolates (Table 3.2) was determined by pairing the *nit 1* and Nit M mutants generated for each of the isolates against a set of known *Focep* VCG testers (Table 3.3). This set included *Focep* VCGs 0421 and SMV 4 collected in Colorado, USA (Swift, *et al*., 2002), as well as VCG 0425 and SMV 3, collected in the Western Cape Province of South Africa (Southwood, 2010). Heterokaryon self-incompatibility was determined by pairing *nit 1* and Nit M mutants of the same isolate with each other. The plates were incubated for 7-10 days at 25°C under florescent light. When the complimentary *nit* mutants from different isolates formed a robust line of mycelial growth on MM (Puhalla, 1985), they belonged to the same VCG.

3.2.4 Screening for virulence factors among *Fusarium* isolates

The presence of virulence factors in isolates listed in Table 3.2 including 19 previously characterised *F. oxysporum* isolates from South Africa and Colorado, USA (Table 3.3), were determined by amplifying genomic DNA with primers of the *SIX3, SIX5,* and *SIX7* genes (Meldrum *et al.,* 2012). These primers produced a clear band when tested on *Focep* strains during a pre-screen experiment where primers for *SIX* genes 1-8 were used for amplification of genomic DNA. Thermocycling conditions were set according to Meldrum (2012) in a reaction volume of 25 µl, which consisted of 12.5 µl KAPA Taq ReadyMix (2X) (Kapa Biosystems, South Africa), 0.5 µl (0.2 µM) of each primer and 2 µl (19.09 µg/ml) genomic DNA. A Veriti® 96-Well Thermal Cycler (Applied Biosystems, South Africa) was used for amplification starting with an initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 45 s, annealing at 60°C for 58 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. Annealing temperatures were adjusted to 58°C for *SIX*3 and *SIX5*.

The PCR amplification of *SIX3, SIX5* and *SIX7* genes were also repeated using the primers designed by Vágány (2012). For this amplification, thermocycling conditions for *SIX7* started with an initial denaturation temperature of 95°C for 5 min, followed by 40 cycles at 93°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, and ending with a final extension at 72°C for 10 min. The initial denaturation for *SIX3* and *SIX5* was at 94°C for 1.5 min, followed by 35 cycles at 94°C for 30 s, annealing at 57°C for 1.5 min, extension at 68°C for 2 min, and a final extension at 72°C for 10 min. The amplicons were separated on agarose gel (0.8%), where after the gel was stained with ethidium bromide to visualise the bands under UV light (Meldrum *et al*., 2012).

3.3 Results and Discussion

3.3.1 *Fusarium* isolations from Vaal River and onion bulbs

In total, 59 single-spore *Fusarium* isolates were obtained from 73 samples of river water collected at 15 sampling sites along a 159 km stretch of the Vaal River (Table 3.1, Table 3.2). Another eight *Fusarium* isolates were acquired from symptomatic and asymptomatic onion bulbs cultivated on a farm in the same region (Table 3.2). Morphological identification revealed four species, namely *F. oxysporum* (52 isolates), *Fusarium solani*, (three isolates), *Fusarium semitectum* (syn., *Fusarium incarnatum*) (one isolate) and *Fusarium subglutinans* (three isolates).

Table 3.1. Isolates tentatively identified as *Fusarium* species, obtained from water sampled in the Vaal River.

Sampling date	[a] CAB number	Volume of water filtered
09-May-11	143-147, 153	2 L
31-May-11	148, 150-151	1.5 L
26-Aug-11	330	1.5 L
30-Nov-11	157	1.5 L
09-Sep-12	323	1 .5 L
08-Dec-12	321-322, 324-325	3 L
01-Feb-13	328-329	1.5 L
20-Feb-13	164, 326, 331, 332-334	3 L
05-Jun-13	335-337	4 L
11-Jul-13	338-353, 355-360, 364, 369	6 L

[a] CAB numbers were allocated to isolates after single spore isolation and identification before depositing in the fungal culture collection of Stellenbosch University, Department Microbiology

Table 3.2. Pathogenicity to onions of *Fusarium* isolates obtained from water in the Vaal River and onions planted on farms next to the river.

[a] Isolate	Origin	Species name	[b] Pathogen (Y / N)	[c] Targeted PCR for *SIX* genes		
				*SIX*3	*SIX*5	*SIX*7
143	Vaal River	*Fusarium solani*	N	-	-	-
144	Vaal River	*Fusarium solani*	N	-	-	-
145	Vaal River	*Fusarium oxysporum*	N	-	-	-
146	Vaal River	*Gibberella fujikuroi*	N	-	-	-
147	Vaal River	*Fusarium oxysporum*	N	-	-	-
148	Vaal River	*Gibberella fujikuroi*	N	-	-	-
150	Vaal River	*Fusarium oxysporum*	N	-	-	-
151	Vaal River	*Fusarium oxysporum*	N	-	-	-
152	Onion	*Fusarium oxysporum*	Y	+	-	+
153	Vaal River	*Fusarium oxysporum*	N	-	-	-
154	Onion	*Fusarium oxysporum*	N	-	-	-
155	Onion	*Fusarium oxysporum*	Y	+	+	+
156	Onion	*Fusarium oxysporum*	Y	+	+	+
157	Vaal River	*Fusarium oxysporum*	N	-	-	-
158	Onion	*Fusarium oxysporum*	Y	+	+	+
164	Vaal River	*Fusarium oxysporum*	N	-	-	-
321	Vaal River	*Fusarium oxysporum*	N	-	-	-
[d]322	Vaal River	*Fusarium oxysporum*	N	-	-	-
323	Vaal River	*Fusarium oxysporum*	N	-	-	-
324	Vaal River	*Fusarium oxysporum*	N	-	-	-
325	Vaal River	*Fusarium oxysporum*	N	-	-	-
326	Vaal River	*Fusarium incarnatum-equiseti*	N	-	-	-
*328	Vaal River	*Fusarium brachygibbosum*	n.d	-	-	-
*329	Vaal River	*Fusarium incarnatum-equiseti*	n.d	-	-	-
330	Vaal River	*Fusarium oxysporum*	N	-	-	-
331	Vaal River	*Fusarium solani*	N	-	-	-
332	Vaal River	*Fusarium brachygibbosum*	N	-	-	-
333	Vaal River	*Fusarium oxysporum*	N	-	-	-
334	Vaal River	*Fusarium brachygibbosum*	N	-	-	-
335	Vaal River	*Fusarium oxysporum*	N	-	-	-

[a] Isolate	Origin	Species name	[b] Pathogen (Y / N)	[c] Targeted PCR for *SIX* genes		
				*SIX*3	*SIX*5	*SIX*7
336	Vaal River	*Fusarium oxysporum*	N	-	-	-
337	Vaal River	*Fusarium oxysporum*	N	-	-	-
338	Vaal River	*Fusarium oxysporum*	N	-	-	-
339	Vaal River	*Fusarium oxysporum*	N	-	-	-
340	Vaal River	*Fusarium oxysporum*	N	-	-	-
341	Vaal River	*Fusarium oxysporum*	N	-	-	-
342	Vaal River	*Fusarium oxysporum*	N	-	-	-
343	Vaal River	*Fusarium oxysporum*	N	-	-	-
344	Vaal River	*Fusarium oxysporum*	N	-	-	-
345	Vaal River	*Fusarium oxysporum*	N	-	-	-
346	Vaal River	*Fusarium oxysporum*	N	-	-	-
347	Vaal River	*Fusarium oxysporum*	N	-	-	-
348	Vaal River	*Fusarium oxysporum*	N	-	-	-
349	Vaal River	*Fusarium oxysporum*	N	-	-	-
350	Vaal River	*Fusarium oxysporum*	N	-	-	-
351	Vaal River	*Fusarium oxysporum*	N	-	-	-
352	Vaal River	*Fusarium oxysporum*	N	-	-	-
353	Vaal River	*Fusarium oxysporum*	N	-	-	-
355	Vaal River	*Fusarium oxysporum*	N	-	-	-
356	Vaal River	*Fusarium oxysporum*	N	-	-	-
357	Vaal River	*Fusarium oxysporum*	N	-	-	-
358	Vaal River	*Fusarium oxysporum*	N	-	-	-
359	Vaal River	*Fusarium oxysporum*	N	-	-	-
360	Vaal River	*Fusarium oxysporum*	N	-	-	-
364	Vaal River	*Fusarium oxysporum*	N	-	-	-
365	Onion	*Fusarium oxysporum*	N	-	-	-
366	Onion	*Fusarium oxysporum*	N	-	-	-
367	Onion	*Fusarium oxysporum*	N	-	-	-
369	Vaal River	*Fusarium oxysporum*	N	-	-	-

[a] CAB numbers of isolates collected from Vaal River water and onions from the Vaal region were deposited in the fungal culture collection of Stellenbosch University, Department Microbiology.
[b] Isolates that caused infection of onion bulbs are marked by Y and non-pathogenic isolates with N
[c] + denotes amplicon detected and - denotes amplicon not detected
* Excluded from pathogenicity tests
[d] CAB 322 did not generate any mutants on chlorate medium

Table 3.3. *Fusarium oxysporum* isolates used as controls in the determination of *SIX* genes and vegetative compatibility status.

Reference isolate	Species name	[e] Pathogen (Y / N)	[d] VCG	[b] Targeted PCR for *SIX* genes		
				*SIX*3	*SIX*5	*SIX*7
Foc 4	[a]*Focep*	Y	0 421	+	+	+
Foc 21	*Focep*	Y	SMV 4	+	+	+
Foc 22	*Focep*	Y	0 424	-	-	-
Foc 47	*Focep*	Y	0 423	-	-	-
STE-U 6636	*Focep*	Y	SMV 1	-	-	-
STE-U 6638	*Focep*	Y	0 426	-	-	-
STE-U 6641	*Focep*	Y	0 425	+	-	+
STE-U 6645	*Focep*	Y	SMV 2	+	+	+
STE-U 6658	*Focep*	Y	SMV 3	-	-	-
STE-U 6666	*F. oxysporum*	N	n.d	-	-	-
STE-U 6675	*F. oxysporum*	N	SMV 5	-	-	-
STE-U 6677	*F. oxysporum*	N	n.d	-	-	-
STE-U 6682	*F. oxysporum*	N	SMV 6	-	-	-
STE-U 6683	*F. oxysporum*	N	SMV 7	-	-	-
STE-U 6684	*F. oxysporum*	N	SMV 8	-	-	+
STE-U 6688	*F. oxysporum*	N	SMV 9	-	-	-
CAV 315	[b]*Fol*	Y	n.d	+	-	-
CAV 317	*F. oxysporum f. sp. melanis*	Y	n.d	-	-	-
CAV 1185	[c]*Foc*	Y	n.d	-	-	-

[a] *Fusarium oxysporum* f. sp*. cepae*
[b] *Fusarium oxysporum* f. sp. *lycopersici*
[c] *Fusarium oxysporum* f. sp. *cubense*
[d] A VCG code consists of a four digit number, the first three digits correspond to *forma specialis*, with the fourth digit designating VCGs within *forma specialis*.
Fusarium oxysporum isolates that do not anastomose with any other isolate, is assigned a single member VCG (SMV) number.
[e] According to Southwood, (2010)
n.d, denotes, not determined
Positive controls are marked Y, while non-pathogens are marked as N.
All isolates were provided by the Department of Plant Pathology culture collection, referred to as STE-U, Foc, and CAV.

3.3.2 Molecular identification of *Fusarium* species

Species identification using morphology was confirmed by BLAST analyses in the FUSARIUM-ID v 1.0 database, with minor exceptions. Amongst the 59 selected isolates, five different *Fusarium* species were identified. The dominant species in both the river water and onions were found to be *F. oxysporum* (99.2% to 100% sequence similarity to *F. oxysporum*) (Table 3.2). This species is associated with asymptomatic and decaying of onion bulbs in South Africa (Southwood *et al*., 2012a). During the current study four more *Fusarium* species were obtained from the water (Table 3.2), which included *F. solani* (98.7% to 100% sequence similarity to *F. solani*), *Gibberella fujikuroi* (teleomorph of *Fusarium moniliforme* with 96.4% to 96.2% sequence similarity to *Gibberella fujikuroi*) (Klittich & Leslie, 1988), *F. incarnatum-equiseti* (98.6% to 100% sequence similarity) and *F. brachygibbosum* (98.6% to 100% sequence similarity to *F. brachygibbosum*).

3.3.3 Pathogenicity testing

Three *F. oxysporum* isolates collected from asymptomatic onion bulbs (CAB 152, 155, 156), as well as one *F. oxysporum* isolate from a symptomatic onion (CAB 158), caused disease symptoms typical of basal rot on inoculated onion bulbs 6 weeks after inoculation. These symptoms include a watery soft decay of the inner bulb, accompanied by purple to brown growth inside halved bulbs (Figure 3.2). Negative control bulbs exhibited no infectious symptoms, and remained symptomless until the end of the experiment. The positive control, a highly virulent *Focep* isolate STE-U 6643, caused severe infection during the experiments. The isolates collected from farms in the Northern Cape Province that caused basal rot to onions, therefore, were identified as *Focep* isolates (Table 3.2; Figure 3.2). This discovery was in contrast to the results obtained by Southwood *et al*. (2012b), who found only non-pathogenic *F. oxysporum* strains collected from onion bulbs in the Northern Cape Province of South Africa. Four *F. oxysporum* isolates from asymptomatic onions (CAB 154 and 365-367) did not infect the onion bulbs on which they were tested. The *Fusarium* spp. isolated from the Vaal River were all non-pathogenic. These isolates could, however, be pathogenic towards a different cultivar of onion if they belong to a different race of *Focep* that does not attack the onion cultivar ‘Lombardi’ (Correll, 1991; Aloi & Baayen, 1993; Leslie & Summerell, 2006; Galván, *et al.,* 2008).

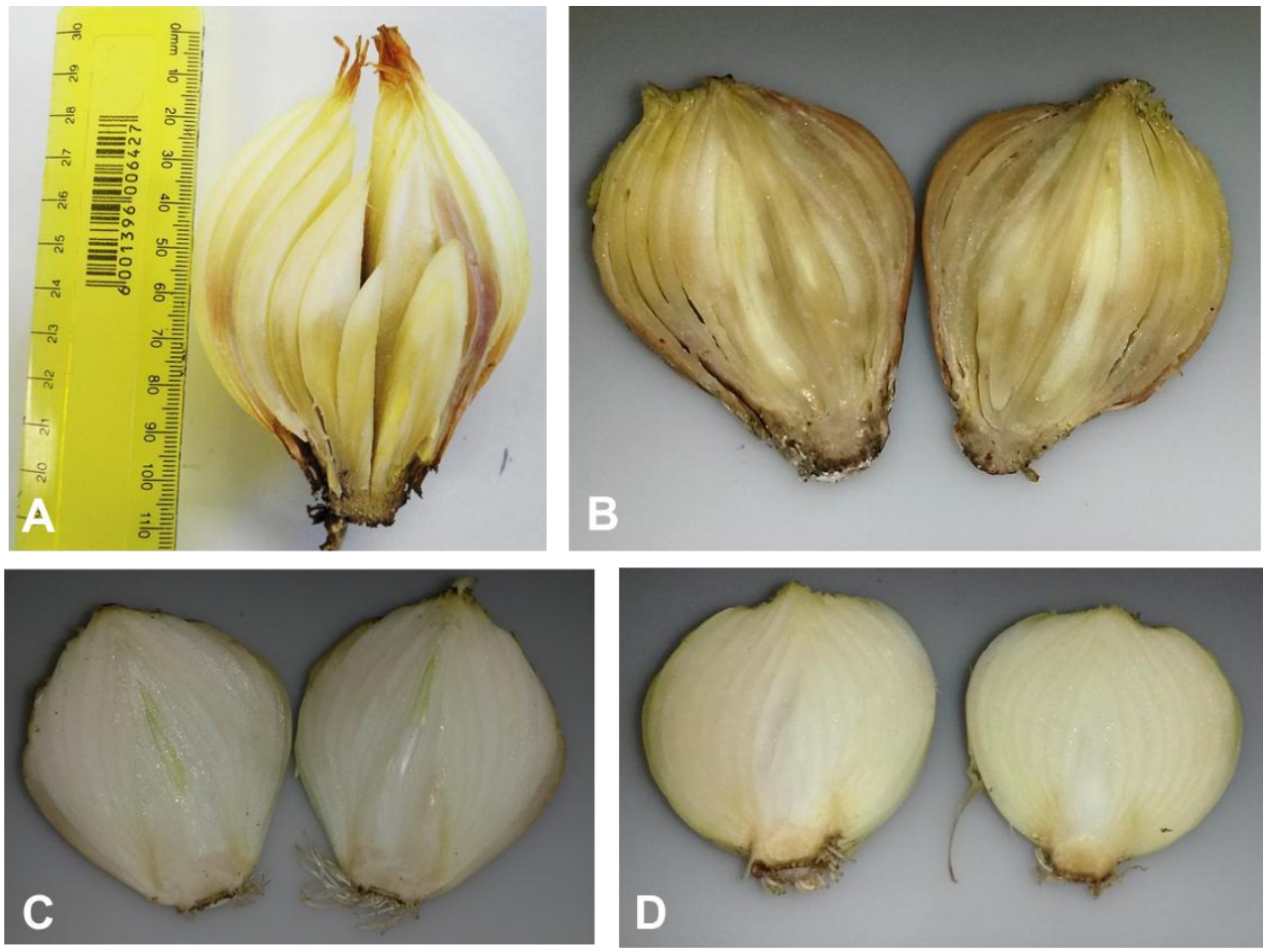

Figure 3.2. Characteristic Fusarium basal rot symptoms caused by *Fusarium oxysporum* f. sp. *cepae* isolates CAB 156 (A) and the positive control STE-U 6643 (B). Asymptomatic onions inoculated with CAB 343 (C) and sterile distilled water (D) as negative controls.

3.3.4 Classification of pathogenic isolates according to vegetative compatibility groups

The four pathogenic *Focep* isolates from onion farms along the Vaal River (CAB 152, 155, 156, and 158) were heterokaryon self-compatible. Of these, isolate CAB 158 formed a stable heterokaryon with VCG 0425. Although CAB 152, 155 and 156 did not form stable heterokaryons with VCG 0425, they anastomosed with CAB 158, indicating that they were indeed members of VCG 0425. The non-pathogenic strains (Table 3.2) did not anastomose with any *Focep,* VCG or SMV tester strains. None of the river isolates were compatible when paired amongst each other or with the *Focep* tester strains.

When genes of isolates from the same species differ at loci governing heterokaryon formation, no heterokaryon formation will take place (Leslie & Summerell, 2006). This finding was supported by Bayraktar *et al.* (2010) who, during a VCG study of *Focep* in Turkey, concluded that heterokaryon formation was prevented by isolates which may have undergone mutations at one or more *vic* loci. The *Focep* isolates CAB 152, 155, 156 and 158 were clones able to pair with each other, fitting them into VCG 0425. This is the second record of VCG 0425 in the Northern Cape (Philippou, 2014). Considering the small number of isolates of *Focep* from the Northern Cape Province tested, it is possible that members of the other seven VCGs (VCGs 0420 to 0426) and several SMVs (Southwood *et al*., 2012b) present in South Africa might also be found in the region.

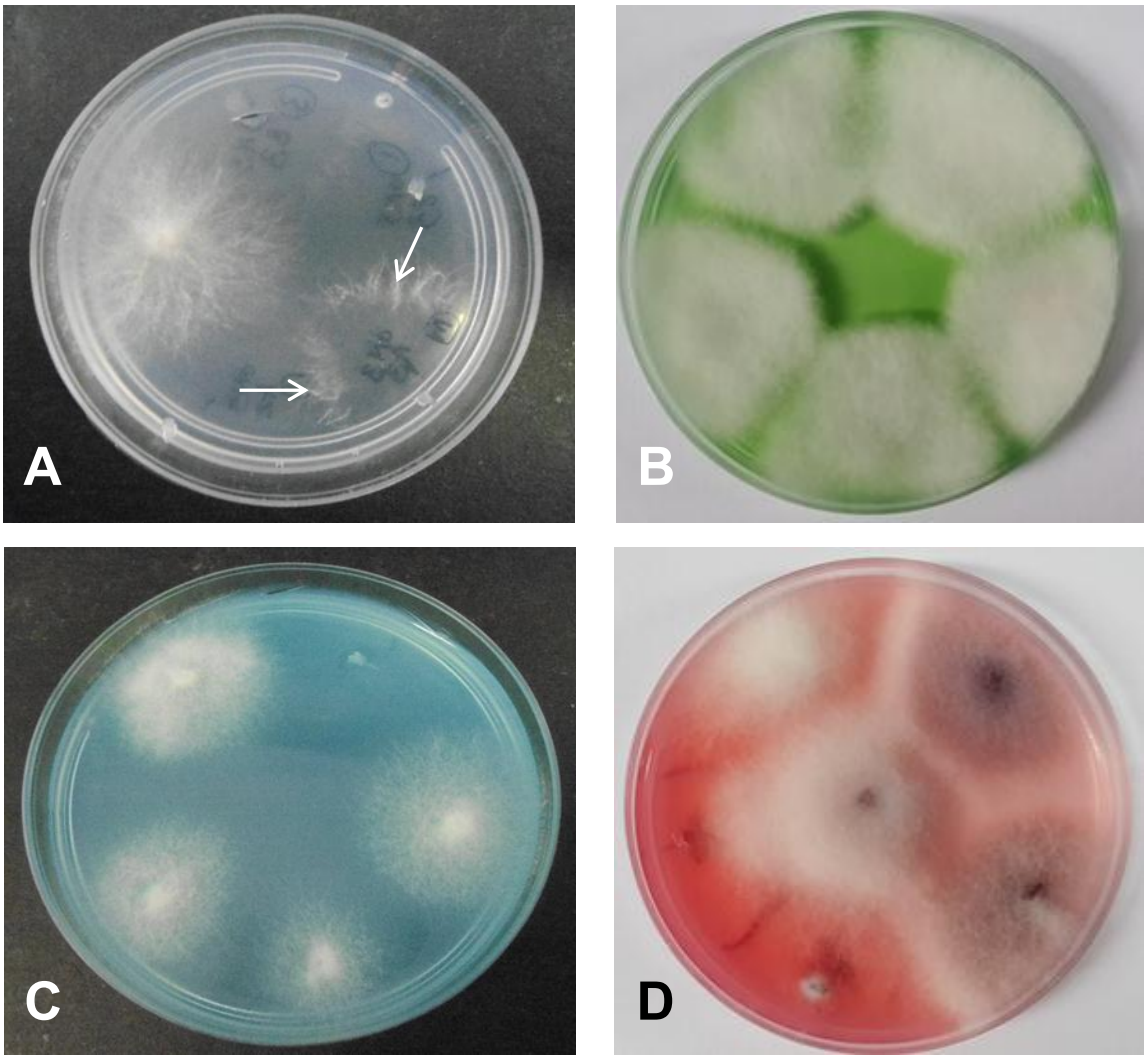

Figure 3.3. Nit M and *nit* 1 mutants of *Fusarium oxysporum* f. sp. *cepae* isolate CAB 152 on minimal media (A). White arrows indicate were anastomoses took place between compatible mutants. Growth of CAB 152 on typing media for classification of mutants, supplemented with NH_4^+ (B), NO_2^- (C), and hypoxantine (D).

3.3.5 Screening for virulence factors among *Fusarium* isolates

Fusarium isolates collected in the Northern Cape Province (Tables 3.2), as well as representatives of non-pathogenic *F. oxysporum*, *F. oxysporum* f. sp. *cepae*, *F. oxysporum* f. sp. *lycopersici*, and *F. oxysporum* f. sp. *cubense* (Table 3.3) were screened for the presence of virulence factors, i.e. *SIX3, SIX5,* and *SIX7,* using primers designed by Meldrum *et al*. (2012) and Vágány (2012).

The *SIX3* gene, of aproximately 600 bp in size, was present in the *F. oxysporum* isolates CAV 315, STE-U 6641, STE-U 6645, Foc 4 and Foc 21 (Table 3.3), and *Focep* isolates CAB 152, 155, 156 and 158 collected in the Northern Cape Province (Table 3.2). Amplification of isolates with the *SIX5* primer pairs resulted in a band aproximately 650 bp in size in isolates Foc 4, Foc 21 and STE-U 6645, and the onion pathogens CAB 155, 156 and 158 (Table 3.2-3.3). *SIX7* genes were confirmed in isolates STE-U 6641, 6645, 6684, Foc 4 and Foc 21, as well as in the onion pathogens CAB 152, 155, 156 and 158. These results were confirmed when isolates were screened with primers designed by Vágány (2012).

In this study, all *Focep* isolates causing basal rot in the onion cultivar 'Lombardi' contained *SIX* genes. However, *SIX3* and *SIX5* genes were absent from *Focep* isolate CAB 152 (Table 3.4). Interestingly, the non-pathogenic control isolates STE-U 6675 and STE-U 6684, as well as non-pathogenic river isolates (CAB 143, 144, 153, 164, 331 and 350) collected during the present study, was shown to harbour the *SIX7* gene (Table 3.4). This suggest that they could potentially be pathogenic to a different plant host or cultivar of onion (Özer *et al.*, 2004; Schwartz & Mohan, 2008; Galván, *et al*., 2008; Chikh-Rouhou *et al*., 2013). The *SIX7* gene could also play a more general role in pathogenicity, a contention made by Vágány (2012), who revealed that *SIX7* shares homologues with *Fol*. Vágány (2012) found that more than one *formae speciales* may occur per clonal lineage within *F.oxysporum.* For example, representatives of *formae speciales cepae, batatas, lycopersici,* including a human pathogenic strain, share identical EF sequences. Likewise, homologues of *SIX7* were previously found in several other *formae speciales* of *F. oxysporum*, including *F. oxysporum* f. sp *cubense* and *F. oxysporum* f. sp *lilii* (Lievens *et al*., 2009; Meldrum *et al*., 2012).

The absence of *SIX* genes from isolates CAB 152 when using the primers of Vágány (2012), and STE-U 6641 when the primers of Meldrum *et al*. (2012) were used, could be due to mutations present in the primer annealing site, causing these genes to not be detected (Vágány, 2012). Vágány (2012) also observed the absence of certain *SIX* genes in highly virulent *Focep* isolates, *SIX3* and *SIX5* were more frequently associated with pathogens than *SIX7*. *Focep* isolates devoid of *SIX7* may have lost the gene in its attempt to overcome plant immunity by mediating its recognition in the plant host (Takken & Rep, 2010). If the *Allium* plant recognises the six7 protein, *Focep* can escape the recognition by losing *SIX7*. Plant immunity may also be overcome by mutating the *SIX* gene to avoid recognition (Vágány, 2012).

In the current study, *Fol* isolate CAV 315 only contained the *SIX3* gene, despite this gene being present in a large number of *Fol* isolates screened by Lievens *et al.* (2009). Lievens *et al.* (2009) believed that *SIX3* and *SIX5* could be used in the unambiguous identification of *Fol*, but this was not the case in the current study. This study was the second to demonstrate that *Focep* isolates contain *SIX* genes that can produce virulence factors.

Table 3.4. Amplification products of *Fusarium oxysporum* with *SIX* gene primers designed by Vágány (2012).

[a] Isolate	[b] Pathogen (Y/N)	*SIX7*				*SIX3*	*SIX5*
		FOC403f-FOC535r	FOC403f-FOC503r	FOC48f-FOC657r	FOC424f-FOC619r		
CAB 143	N	-	-	-	+	-	-
CAB 144	N	-	-	+	-	-	-
CAB 152	Y	-	-	+	+	-	-
CAB 153	N	-	-	+	+	-	-
CAB 155	Y	+	+	+	+	+	+
CAB 156	Y	+	+	+	+	+	+
CAB 158	Y	+	+	+	+	+	+
CAB 164	N	-	-	-	+	-	-
CAB 331	N	-	-	-	+	-	-
CAB 340	N	-	+	-	-	-	-
CAB 350	N	-	-	-	+	-	-
Foc 4	Y	+	-	+	+	+	+
Foc 21	Y	-	+	+	+	+	+
STE-U 6658	Y	-	-	-	-	+	-
STE-U 6641	Y	+	-	+	+	+	+
STE-U 6645	Y	+	-	+	+	+	+
STE-U 6675	N	-	-	-	+	-	-
STE-U 6684	N	-	-	+	-	-	-

[a] Only isolates positive for *SIX* genes i.e. reference and Vaal River waterborne isolates, are listed
[b] Isolates that caused infection of onion bulbs are marked by Y and non-pathogenic isolates with N

3.4 Conclusion

Irrigation water from the Vaal River contained several *Fusarium* species, but none of these were pathogenic to onion bulbs. Four *Focep* isolates collected from onions in the Northern Cape Province belong to VCG 0425. This is the second time that VCG 0425 had been found in onion fields in this area. Philippou (2014) used a *Focep* isolate collected from Barkley West, as a control during a study aimed at characterising the *F. oxysporum* pathogen causing wilt rot in *Hoodia gordonii*. Southwood (2010) reported that VCG 0425 resulted in severe losses on farms in certain Western Cape areas. Since this VCG was not found in the irrigation water from the Vaal River during the current study, it is tempting to speculate that it was introduced into the Northern Cape Province with infected seed or seedlings originating from the Western Cape Province. Onion seedlings originating from commercial nurseries are distributed countrywide in South Africa (Western Cape Government, 2011).

Although *Fusarium* isolates from the Vaal River did not cause disease to onion, other produce cultivated in the area may still be at risk of infection. Screening the waterborne *Fusarium* isolates for *SIX* genes revealed that a number of these isolates contain virulence factors. It is therefore suggested that these isolates be inoculated onto more potential hosts cultivated in the area in future, such as watermelon and potato. Since *Fusarium* species collected from the Vaal River contain potential plant pathogens, it is recommended that the water is treated with a disinfectant to reduce pathogen levels before it is used to irrigate crops.

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Chapter 4

Effectivity of a calcium hypochlorite containing disinfectant in the removal of faecal coliforms and *Fusarium* from the Lower Vaal River

4.1 Introduction

The Vaal River Barrage is known to be subjected to unacceptably high levels of faecal pollution (Chapter 2; Tempelhoff, 2009; Botes, 2007; Seale, 2007). However, it is not only sewage, represented by total and faecal coliforms, which pollute river systems and lead to ecosystem degradation. Excess plant pathogens (e.g., *Fusarium* spp.), also occur in water sources, resulting in a threat to crop production (Solomon, 2002; Steele & Odumeru, 2004; Steele, *et al.,* 2005). Farmers that use water from the Vaal River for irrigation purposes are therefore concerned about the quality thereof (Chapter 1.4; Hameed *et al*., 2008; López-Mondéjar *et al*., 2012). A need thus arose to treat the river water, to reduce pathogen levels, before it is dispersed onto crops via irrigation systems.

Both physical and/or chemical methods are used to treat water sources in order to render it suitable for agricultural applications or human consumption (Chapter 1.6.2; Binnie *et al*., 2002). These methods usually include filtration, and/or treatment with ozone, ultraviolet light and chlorine (Chapter 1.6.2; Edberg *et al*., 2000; APHA *et al*., 2012). However, the most widely used chemical method to disinfect water is treatment with chlorine. The most common form of chlorine is calcium hypochlorite which is used to chlorinate swimming pools and irrigation water (Hong *et al*., 2003). Generally calcium hypochlorite contains 65% to 70% of available chlorine (WHO, 2008; WHO, 2011). The efficacy of this chlorine however, may vary depending on the physical and biological properties of the water that is being treated e.g., pH, temperature, turbidity and microbial load (WHO., 2005; WHO, 2008; Migliaccio *et al*., 2009; WHO, 2011). It is therefore important to first test the efficiency of chlorine containing disinfectants against potential pathogens in conditions similar to the situation on the farm, before applying the disinfectant via an irrigation system.

A typical irrigation system on a commercial farm is the centre-pivot irrigation system (Figure 4.1). In such systems, water is pumped directly from a river, or another water source, along a set of pipes, directed towards the hub of the centre-pivot. Sixteen seconds after reaching the hub water is released through the first set of sprinklers, while it may reach the final set of sprinklers, depending on the size of the system, after ca. 360 s. To treat water at the hub of such an irrigation system, before it is dispersed by the system, the concentration of chlorine must be enough to be effective within 16 s, but not be too high, as to damage agricultural crops (personal communication with farmers; Nel *et al*., 2007).

With the above as background the first aim of this study was to determine the efficacy of a calcium hypochlorite containing disinfectant, 'HTH® Super Shock It', to remove coliform bacteria and *Fusarium* spores from samples of river water, differing in pH, turbidity, and temperature, within 16 s, 360 s and 3600 s.

The second aim was to find correlations between the physico-chemical properties of the water and the efficacy of the commercial disinfectant and finally, to identify physical and/or chemical components in the water which may affect the efficacy of the disinfectant.

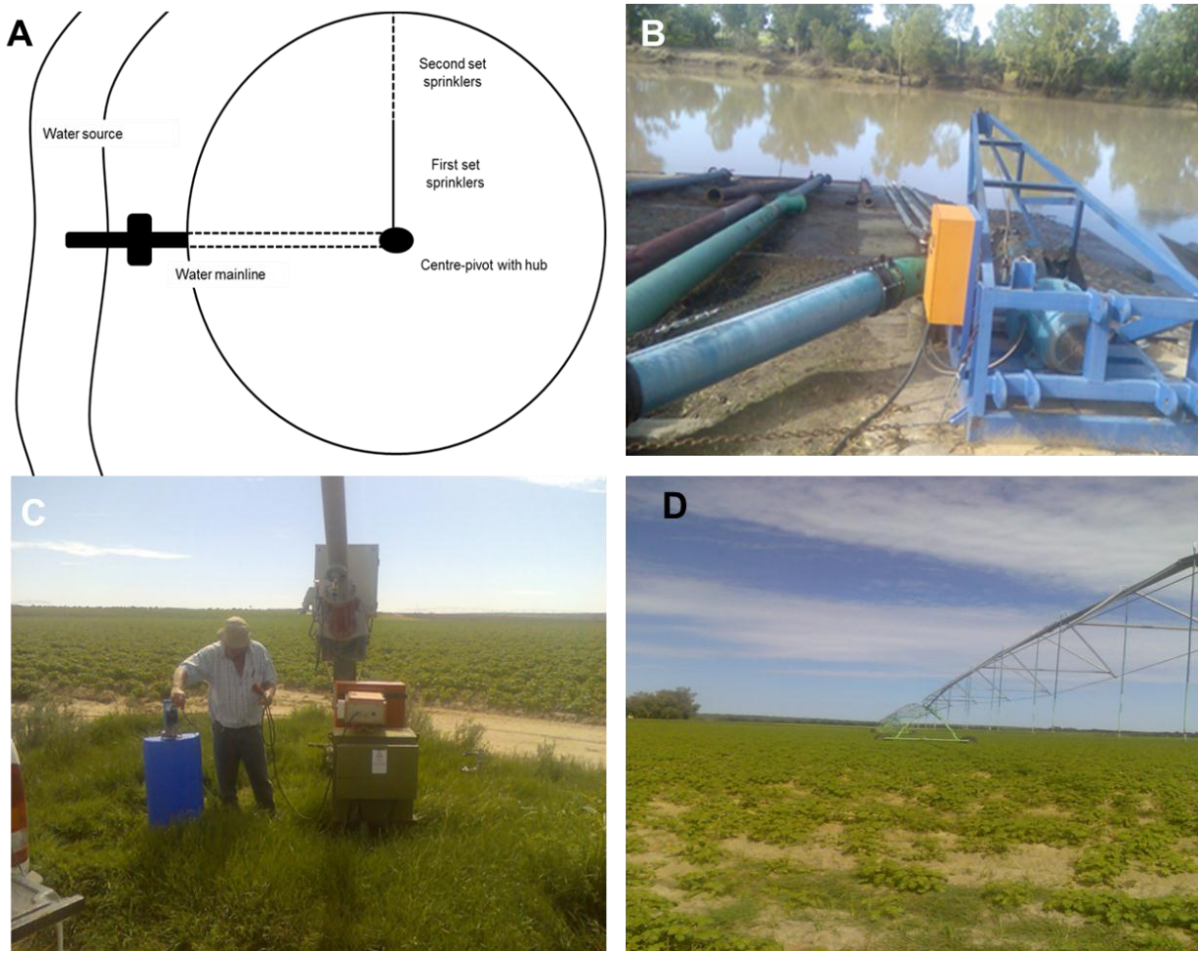

Figure 4.1. General layout of a centre-pivot irrigation system. River water is pumped along a mainline (A, B) towards the centre-pivot containing the hub (C). After 16 s the first set of sprinklers release water, while after 360 s the last set release water (D).

4.2 Materials and Methods

4.2.1 Determining the effective concentration of 'HTH® Super Shock It' against coliforms, and *Fusarium* spores

4.2.1.1 Analyses of water samples

Within 24 h after taking samples of faecal contaminated water (obtained from undisclosed locations along the Vaal River; Chapter 2, Table 2.1) sub-subsamples were used for disinfection trials against *Escherichia coli* and other faecal indicator organisms. The remainder of each sample was aliquoted (500 ml) into sterile Schott bottles and stored at 4°C.

Within one week, the stored water was used in disinfection trials against *Fusarium* spores. For this purpose the stored water samples was autoclaved (121°C, 20 min, 1.5 kg/cm^3) and allowed to equilibrate to ca. 20°C prior to being inoculated with a specific concentration of *Fusarium* spores, and treated with different concentrations of the commercial disinfectant 'HTH® Super Shock It'. The pH and turbidity of each sample was measured before experiments were conducted. Turbidity and the pH of the water was analysed using a portable turbidimeter (Eutech instruments TN-100, Singapore) and bench pH meter (Martini Instruments, Mi 150, U.S.A).

4.2.1.1.1 Disinfection trials against *E. coli* and other faecal indicator organisms

'HTH® Super Shock It' (active ingredient: calcium hypochlorite 700 g/kg) was added to 500 ml aliquots of faecal contaminated river water, to a final concentration of 0.75 mg/L, 1.50 mg/L, 3.13 mg/L, 6.25 mg/L and 15.00 mg/L river water. After an exposure time of 16 s, 360 s, and 3600 s (vigorous agitating throughout) 0.1 ml of the treated water was plated onto both MacConkey agar (Merck, Darmstadt, Germany) and lauryl tryptose agar (Atlas, 1993).

Plates were incubated at 44°C for 24 hours; where after bacterial colonies were enumerated to establish the number of colony forming units (CFU) per 1 ml of treated water. Controls, which received no 'HTH® Super Shock It', were included in the experimentation, while all experiments were conducted in triplicate. The percentage decline in bacterial numbers, as a result of the treatment with 'HTH® Super Shock It', was calculated for both the results obtained on the MacConkey agar and the lauryl tryptose agar plates.

4.2.1.1.2 Disinfection trials against *Fusarium* spores.

Water samples were inoculated with a specific concentration of *Fusarium* spores. For this purpose, a fungal spore inoculum was obtained by first growing single spore cultures of *Fusarium oxysporum* (CAB 154, 156, 157) and *F. solani* (CAB 158), isolated from the Vaal River, on PDA (potato dextrose agar; Merck, Darmstadt, Germany) supplemented with 0.5 g/L streptomycin sulphate and 0.2 g/L chloramphenicol (PDA*) for 14 days at 26°C. Spore suspensions were made by flooding PDA* plates twice with sterile 9 ml deionized water and collecting the suspension in a sterile 20 ml McCartney bottle. Aliquots (1 ml each) of this spore suspension was used to inoculate 500 ml quantities of sterile autoclaved (121°C, 15 min) river water, amounting to ca. 800 spores per ml.

'HTH® Super Shock It' was subsequently added to the inoculated 500 ml aliquots of sterile river water, to a final concentration of 3.13 mg/L, 4.50 mg/L, 6.25 mg/L and 7.50 mg/L of river water. Spores were exposed to chlorine for 16 s, 360 s, and 3600 s (vigorous agitating throughout); where after 0.1 ml of the disinfected water was plated out onto PDA* plates. Plates were incubated at 26°C for 4-5 days following enumeration of fungal colonies to establish the number of CFU per 1 ml of treated water. Controls, which received no 'HTH® Super Shock It', were included, with all experiments conducted in triplicate. The percentage decline in fungal spore numbers, as a result of the treatment with 'HTH® Super Shock It', was calculated for the results obtained on the PDA* plates (Addendum, Table 1).

To obtain an indication of the residual concentrations of chlorine in the disinfected irrigation water, the average chlorine residuals were determined at 16 s, 360 s and 3600 s after treatment with 4.50 mg/L and 7.50 mg/L 'HTH® Super Shock It'. Chlorine residuals were tested for according to Method 4500-Cl G (APHA *et al*., 2012).

4.2.2 Inorganic and organic composition of river water

An estimation of the inorganic components of the river water was obtained by first preparing a 1.6 L composite sample, comprising of sixteen 100 mL subsamples, of the water taken at Barkley West (Sampling Sites S1, S3, S4, S5) on 20 February 2013 and 5 June 2013 (Chapter 2, Table 2.1). Suspended solids were separated from the river water via filtration (diameter, 0.22 µm), where after the solids were freeze dried and the inorganic components thereof analysed using X-ray diffractometry (XRD, Bruker AXS D8 Adva1nce powder diffractometer with Cu Kα radiation) and a Niton XL3t hand held XRF elemental analyser. The presence of minerals was confirmed using XRD, while the XRF elemental analyser was used to determine relative concentrations of the main elements occurring in the suspended solids (Yariv & Lapides, 2005; Schaef, *et al*., 2012; Vaggelli & Cossio, 2012).

To obtain an indication of the chemical oxygen demand (COD) of the river water, three samples (Chapter 2, Table 2.1) taken at Barkley West on 5 June 2013 (two at Sampling Site S3, and one at S5), were analysed using a photometric kit (Spectroquant®; Merck-Millipore) according to the manufacturer's instructions. All measurements were done at least in triplicate.

4.2.3 Influence of inorganic suspended solids and COD on chlorine efficacy

The main inorganic components of the suspended solids in the river water were respectively suspended in 1 L sterile water (pH 8.85, NaOH). These suspensions either contained the clay mineral smectite (STX-1; 21.1 mg/L), the iron-oxide mineral goethite (1.61 mg/L) or 36 mg/L sieved (53 µm, diameter) quartz. Each of these suspensions were subsequently inoculated with 1 ml spore suspension of *Fusarium oxysporum* CAB 164, prepared as previously described in section **4.2.1.1.2**., amounting to a final concentration of 700 spores per ml. These 1 L suspensions were then respectively treated with 4.50 mg/L and 7.50 mg/L 'HTH® Super Shock It'. The control contained only distilled water and the disinfectant, with no added suspensions. After 360 s and 3600 s of exposing the fungal spores to chlorine in the different suspensions, plate counts were used to enumerate culturable fungal spores on PDA* agar. Colonies were counted after 4-5 days of incubation at 26°C.

The effect of organic matter (60 mg/L COD) on the efficacy of 'HTH® Super Shock It' was determined in a solution containing 0.056 g/L glucose. The latter concentration was selected from calculations and values provided in literature (van Haandel & van der Lubbe, 2012). Thus, 1 L aqueous solution containing 0.056 g/L glucose was inoculated with 1 ml spore suspension of *Fusarium oxysporum* CAB 164, prepared as previously described in section **4.2.1.1.2**., amounting to a final concentration of ca. 700 spores per ml. The 1 L spore suspension was then treated with 7.50 mg/L 'HTH® Super Shock It'. The control contained inoculated distilled water and 7.50 mg/L 'HTH® Super Shock It'. After 360 s and 3600 s of exposing the fungal spores to the chlorine disinfectant, plate counts were used to enumerate culturable fungal spores on PDA* agar. Colonies were counted after 4-5 days of incubation at 26°C.

4.2.4 Statistical analysis

Calculation of means and standard errors for continuous variables where performed using Microsoft Excel Office, version 2007. Functional relationships between data for percentage decline in CFU over pH, turbidity, time, and chlorine concentration, were analysed using correlation matrixes. Univariate test of significance (ANOVA) were also performed on the data and was also subjected to the Bonferroni post hoc test and Mann-Whitney U test. Non-parametric correlation analyses were done using Spearman rank order correlations. Significant differences and correlations were calculated at the 5% significance level. Statistical analyses were computed using STATISTICA version 11 (http://www.statsoft.com; Statsoft, Inc., Tulsa, Oklahoma, USA).

4.3 Results and Discussion

4.3.1 Determining the effective concentration of chlorine against coliforms and *Fusarium* spores

Chlorination is known as the most efficient and cost effective disinfection method, and has been used as such for decades (Tully, 1914; Edberg *et al.*, 2000; APHA *et al.*, 2012). Disinfection by chlorine is one of non-selective oxidation; reacting with a variety of cellular components, effecting metabolic processes of microorganisms (Shang, 1999). With its primary objective in wastewater treatment being, deactivation of disease causing microorganisms (APHA *et al*., 2012).

Experiments, to determine the appropriate chlorine concentration to remove coliforms and *Fusarium* spores in the river water, were designed so that the results would be applicable to a centre-pivot irrigation system used by many farmers along the banks of the Vaal River. It was planned that before dispersal, irrigation water would be treated with chlorine at the hub of the centre-pivot. The chlorine concentration thus had to be effective within 16 s, but low enough not to damage crops (personal communication with farmers; Nel *et al*., 2007). The lowest concentration of 'HTH® Super Shock It' that removed more than 99% of faecal coliforms after 16 s, 360 s and 3600 s was found to be 1.50 mg/L (Table 4.1). The concentrations of 'HTH® Super Shock It' used to treat the contaminating faecal coliforms in the river water were found to be less effective against *Fusarium* spores (Table 4.2).

Thus, the dosage had to be increased. This supported other findings, which mention if concentrations greater than recommended were used, the efficacy of chlorine containing disinfectants increased against *F. oxysporum* f.sp *cubense* spores (Nel *et al*., 2007). We found that at a 'HTH® Super Shock It' concentration of 4.50 mg/L, a relatively small decline of 13.22% and 14.89% was observed after 16 s and 360 s respectively, with 100% of spores destroyed after 3600 s (Table 4.2). At a higher 'HTH® Super Shock It' concentration, i.e. 6.25 mg/L, about 10%, 37% and 100% spores were destroyed after 16 s, 360 s and 3600 s respectively. An even higher concentration (7.50 mg/L) was subsequently tested, which was found to be more effective, and removed 11.89%, 89.22% and 100% of spores after 16 s, 360 s and 3600 s respectively. This demonstrated that, similar to the results of others, lengthening of the exposure time to a chlorine containing disinfectant might increase removal of waterborne pathogens (Copes, *et al*., 2004; Nel, *et al*., 2007). Thus, whether being added to reach an initial concentration of 4.50 mg/L or 7.50 mg/L in the river water, the 'HTH® Super Shock It' eventually destroyed all the *Fusarium* spores.

To obtain an indication of the residual concentrations of chlorine in the disinfected irrigation water, that would come in contact with the crops, when the river water is treated with 4.50 mg/L and 7.50 mg/L 'HTH® Super Shock It', the average chlorine residuals were determined for 16 s, 360 s

and 3600 s after treatment (Table 4.3). A notable amount of residual chlorine was observed in the water after all three time periods when the water was treated with 7.50 mg/L 'HTH® Super Shock It'. However, the residual chlorine concentration in the water treated with 4.50 mg/L 'HTH® Super Shock It' reached acceptable levels after 360 s and 3600 s, being ca. 0.50 and 0.20 mg/L respectively. This is in accordance with the regulations of WHO (2005) regarding drinking water, which states that the levels of residual chlorine should be between 0.50 and 0.20 mg/L to eliminate any pathogens that my enter water after initial disinfection.

Table 4.1. Percentage decline of coliforms in the presence of different concentrations of chlorine (in the form of 'HTH® Super Shock It'). MacConkey agar was used to enumerate *Escherichia coli* and faecal indicator organisms, while lauryl tryptose agar was used to count *E. coli* and other Gram negative bacteria.

		% Decline over time		
Concentration of chlorine	**Medium**	**16 s**	**360 s**	**3600 s**
15 mg/L	M	100 (0.00)	100 (0.00)	100 (0.00)
15 mg/L	L	100 (0.00)	100 (0.00)	100 (0.00)
6.25 mg/L	M	100 (0.00)	100 (0.00)	100 (0.00)
6.25 mg/L	L	100 (0.00)	100 (0.00)	100 (0.00)
3.13 mg/L	M	99.98 (0.01)	100 (0.00)	100 (0.00)
3.13 mg/L	L	100 (0.00)	100 (0.00)	100 (0.00)
1.5 mg/L	M	100 (0.00)	100 (0.00)	100 (0.00)
1.5 mg/L	L	100 (0.00)	100 (0.00)	100 (0.00)
0.75 mg/L	M	97 (0.67)	34 (6.64)	79 (4.04)
0.75 mg/L	L	82 (7.53)	76 (7.80)	77 (4.60)

M, denotes MacConkey agar; L, denotes Lauryl tryptose agar (Values are the means of three repetitions while the numbers in brackets indicate standard errors).

Table 4.2. Percentage decline of *Fusarium* spores in the presence of different concentrations of chlorine using potato dextrose agar (PDA*) for enumeration.

Concentration of chlorine	Medium	% Decline over time		
		16 s	360 s	3600 s
7.5 mg/L	PDA	11.89 (3.98)	89.22 (4.41)	100 (0.00)
6.25 mg/L	PDA	10.25 (6.08)	37.33 (11.10)	100 (0.00)
4.5 mg/L	PDA	13.22 (7.48)	14.89 (6.27)	100 (0.00)
3.13 mg/L	PDA	6.90 (4.39)	3.38 (3.38)	99 (1.00)

PDA, denotes Potato dextrose agar (Values are the means of three repetitions while the numbers in brackets indicate standard errors)

Table 4.3. Changes in average chlorine residuals over time. Assayed by the DPD colorimetric method. Two concentrations of active chlorine were used for this assay.

Time	Chlorine concentration over average chlorine residual in mg/L	
	7.50 mg/L	4.50 mg/L
16 s	1.17 (0.28)	0.99 (0.34)
360 s	1.02 (0.30)	0.52 (0.06)
3600 s	0.51 (0.28)	0.20 (0.03)

Values are the means of three repetitions while the numbers in brackets indicate standard errors while the numbers in brackets indicate standard errors

4.3.2 Determining the effect of time, temperature, pH and turbidity on chlorine disinfection of *Fusarium* spores

Turbidity was analysed and readings ranged between 22.8 and 271.0 ntu, with an average of ca. 44 ntu (Addendum A, Table 1). The pH of the water measured between 7.87 and 9.48, with an average of ca. pH 8.80.

ANOVA analysis (Figure 4.2) revealed that increased concentrations of chlorine, measured as 'HTH® Super Shock It', resulted in enhanced decline ($p = 0.000$) of culturable *Fusarium* spore numbers in the water. This was especially evident after 360 s. After 3600 s however, no differences were observed between the treatments.

At temperatures ranging between 20°C and 23°C a positive correlation was observed between temperature and percentage decline of culturable *Fusarium* spores when 'HTH® Super Shock It' was used for disinfection.

These results were supported by Spearman rank analysis (R = 0.211; p = 0.028). Interestingly, this increased efficacy of chlorine disinfectants at higher temperatures have been reported by others in literature (Servais *et al.*, 1994; WHO, 2008). However, at the temperature range mentioned above, a negative correlation was found to exist between pH and percentage decline of culturable *Fusarium* spores in the water for 4.50 mg/L, 6.25 mg/L and 7.50 mg/L 'HTH® Super Shock It' (R = -0.480, p = 0.011; -0.4540, p = 0.005 and -0.4424, p = 0.021 respectively). These results support the findings of others, who found chlorine disinfectants to be more effective in slightly acidic water (e.g. pH 5) compared to alkaline solutions (pH 8 to 9) (Copes *et al*., 2004; Migiliaccio, 2009).

Interestingly, in the presence of 'HTH® Super Shock It' a significant positive correlation was observed between the percentage decline of culturable *Fusarium* spores and river water turbidity. This positive correlation was supported by Spearman rank analysis (R = 0.280; p = 0.001). These findings were in contrast to existing literature, which states that as water turbidity increases more chlorine is needed to disinfect the water effectively (Edberg, *et al.* 2000; WHO, 2008).

It was demonstrated in a previous study that chlorine diffuses into wastewater particles (pH, ca. 7; COD, ca. 75 mg/L) by means of a serial two-step process (Dietrich *et al.*, 2003). Chlorine was shown to penetrate through both macroporous and microporous regions of wastewater particles, and it was suggested that chlorine application could be tailored to inactivate a desired amount of particle-associated microorganisms. Depending on the particle-size distribution within a particular water source, microbial inactivation was achieved at an appropriate contact time and chlorine concentration (Dietrich *et al.*, 2003; Dietrich *et al.*, 2007). It thus seemed that the interactions observed, in this study, between chlorine and suspended particulate matter, responsible for the water turbidity, were more complex than anticipated. This raised the question as to the particle composition and COD of the Vaal River.

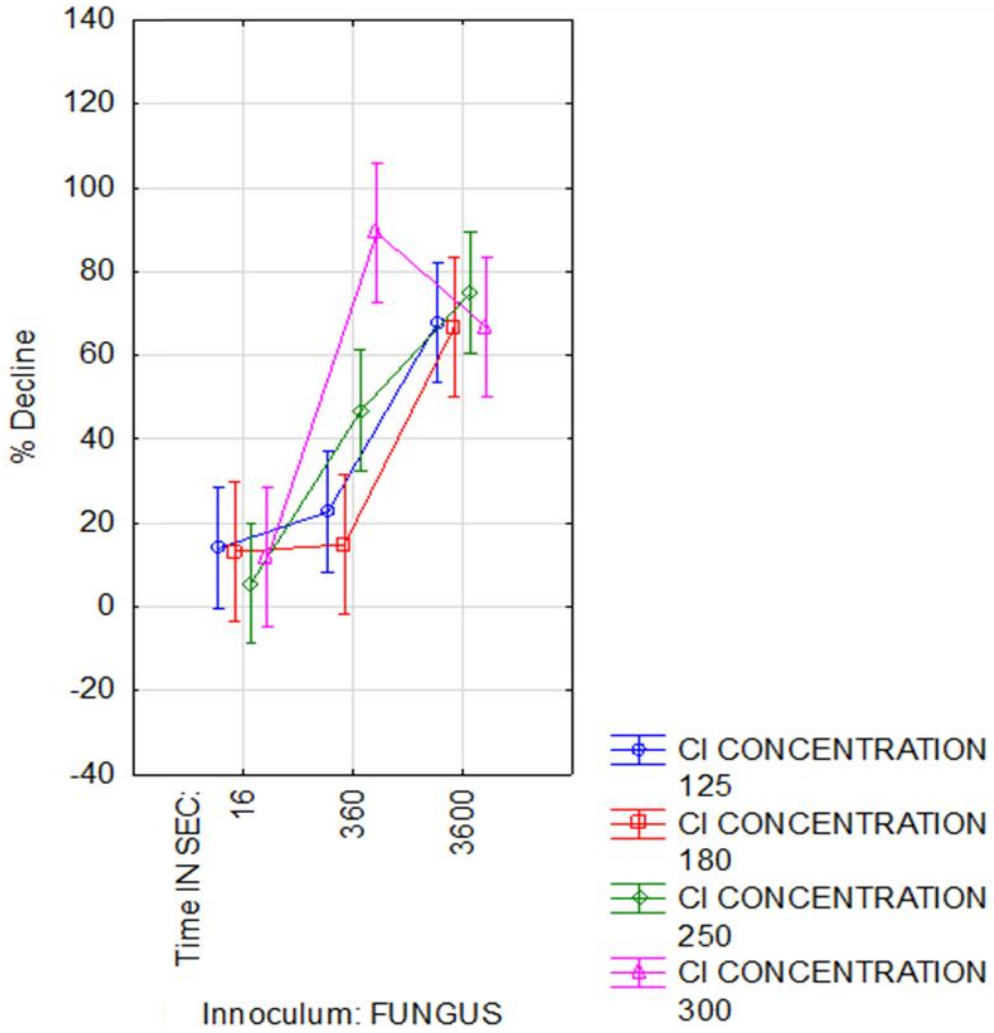

Figure 4.2. Correlation between decline of *Fusarium* spores and time using the efficacy of chlorine, by ANOVA analysis. Vertical bars denote 0.95% confidence level.

4.3.3 Organic and inorganic composition of river water

The concentration of organic compounds, measured as COD, was found to be ca. 60 mg/L. Analyses of the suspended mineral component of the particulate matter using X-ray diffractometry (XRD) revealed the presence of the following; the feldspar albite, the silicate olivine, quartz, and a smectite clay called montmorillonite (bentonite). The results obtained when the main inorganic components were measured using the XRF elemental analyser (Table 4.4), supported the findings of the XRD (Figure 4.3). Both measurements indicated that quartz, i.e. SiO_2, was the chief component of the suspended particulate matter. In addition, both analyses indicated that albite and smectite, together containing aluminium, calcium and silicon, occurred in notable quantities in the particulate matter. The presence of olivine may have been indicated by the iron and silicon that was detected using the XRF elemental analyser (Moore & Reynolds, 1989; Deer *et al.,* 1992).

Table 4.4. XRF results of elemental composition of clay particles from Vaal River.

Element	wt (%)	Standard error
Fe	1.613	0.061
Ca	0.873	0.017
K	1.181	0.107
Al	2.960	0.168
Si	22.98	2.120
SiO_2	49.18	4.537
S	0.244	0.053

wt (%), denotes mass fraction (Values are the means of four repetitions)

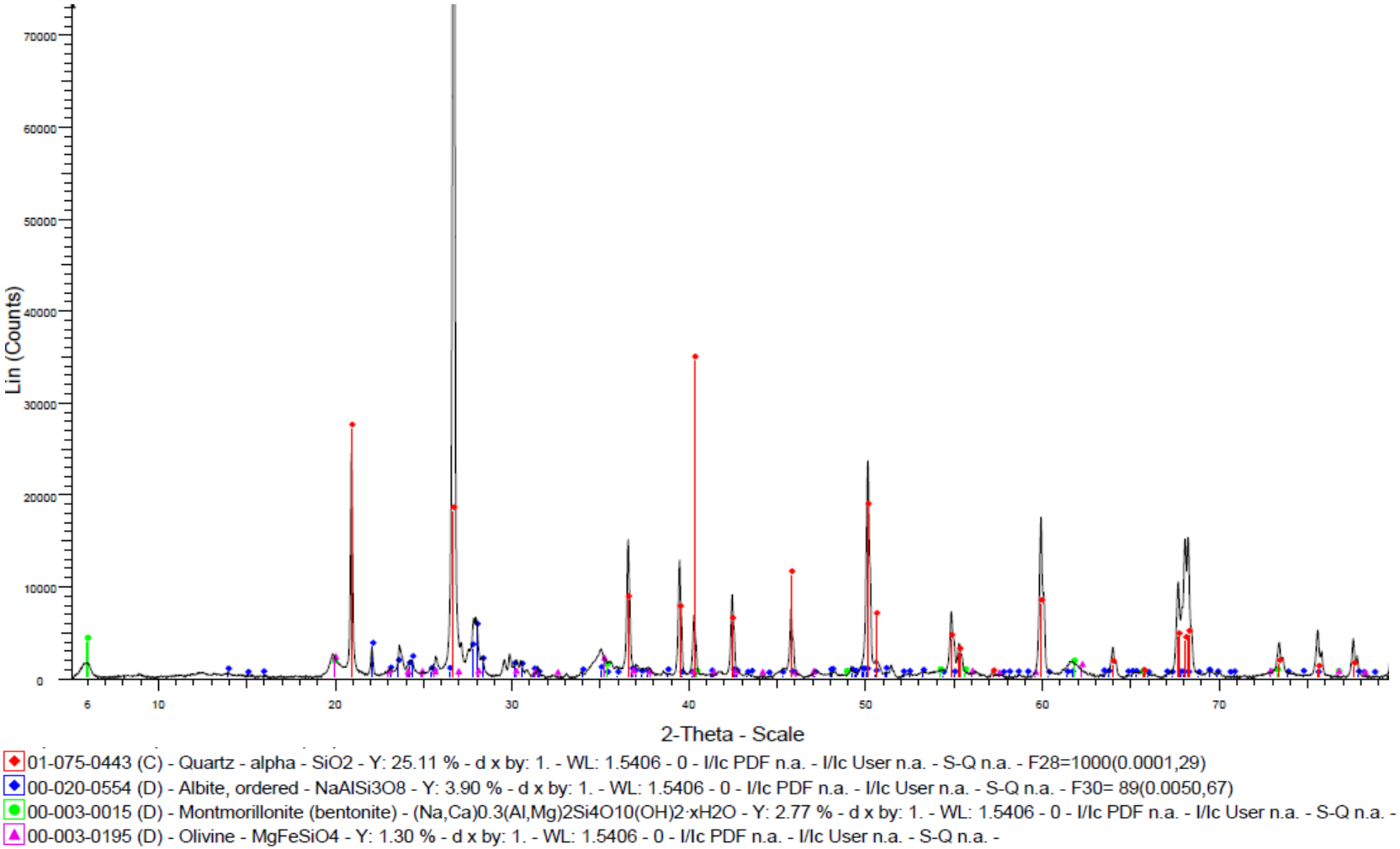

Figure 4.3. X-ray diffraction pattern of the Vaal River clay particles. Measured at room temperature (25°C). Red square bars denote presence of quartz. Blue shows presence of albite. Green square bars denote presence of montmorillonite (smectite). Pink square bars denote olivine.

4.3.4 Effect of inorganic suspended particulate matter and dissolved organic matter on chlorine disinfection of *Fusarium* spores

When a calcium hypochlorite disinfectant such as 'HTH® Super Shock It' is added to water, hypochlorous acid is formed releasing its oxygen in the presence of organic matter. It is this free oxygen, which conducts disinfection by oxidation (Tully, 1914).

Smectite clays are known catalysts for redox reactions on their surfaces (Thompson & Moll, 1973; Laszlo, 1987; Deer *et al*, 1992; Schoonheydt, 2002). In addition, literature states that the zeta potential of smectite is relatively stable over a large pH range, compared to that of albite, olivine, and quartz (Pokrovsky *et al*., 2000; Kaya & Yukselen, 2005; Filippov *et al*., 2012). Thus, to determine whether the smectite in the water may have contributed to the removal of *Fusarium* spores by 'HTH® Super Shock It', the efficiency of this chlorine containing disinfectant in the presence of smectite was compared to its efficiency in the presence of other minerals that may occur in river water. The latter included quartz, known to be chemically inert (Lawrence *et al*., 2003). In addition, an iron oxide mineral known to be abundant in nature, i.e. goethite, was also included in the experimentation. Goethite has a high specific surface area and has been studied in water treatment processes to remove organic pollutants (Shen *et al*., 2006; Garrido-Ramírez *et al*., 2010).

Results, calculated in Microsoft Excel Office, showing the effect of 'HTH® Super Shock It' on the survival of culturable *Fusarium oxysporum* CAB 164 spores, in distilled water suspensions of smectite (STx-1), goethite or quartz, are provided in Figure 4.4. Results were also subjected to ANOVA and Bonferroni post hoc analysis, where it was observed that after 3600 s no significant differences ($p = 1.00$) were observed between the control and any of the pure phase suspensions (i.e. smectite, goethite or quartz). Similarly, ANOVA analysis showed that 360 s after treating the spores with 'HTH® Super Shock It', no significant difference in percentage decline of viable spores were observed between the control and any of the pure phase suspensions. It must be noted however, that 360 s after treating the suspensions with 7.50 mg/L 'HTH® Super Shock It' viable spores in the presence of goethite and smectite tended to be less than the spores suspended in distilled water or in the quartz mixture (Figure 4.4.).

The perceived enhanced reduction in culturable spores, as a result of the disinfectant, in the presence of goethite and smectite, may have been as a result of the surface chemical properties of these minerals. However, the geometry or surface morphology (pore size) may also affect the accessibility of a potential adsorbate and the reactivity of a clay mineral (Seri-Levy & Avnir, 1991; Malekani, Rice & Lin, 1996).

In addition, smectite has a high cation exchange capacity (Murray, 2000) and it is known that the exchangeable cations absorbed between unit layers, and edges, balances the net positive charge deficiency of this clay (Mayer,1994). Taking in consideration the large surface area of smectite (Murray, 2000), and the ability of this clay mineral to adsorb anions despite its net negative zeta potential (Lagaly *et al*., 1985; Chorover *et al*., 2001), negatively charged fungal spores (Wargenau *et al*., 2011) may have been oxidised by chlorine on the clay's surface during the experimentation. Such surface reactions may also have been responsible for the oxidation of the fungal spores in the presence of goethite, known for its positive surface charge and adsorption of anions (Chorover *et al*., 2001).

Results pertaining to the effect of 'HTH® Super Shock It' on the survival of culturable *Fusarium oxysporum* CAB 164 spores, in water containing glucose, amounting to a COD of 60 mg/L, in the presence of 7.50 mg/L 'HTH® Super Shock It' for 360 s, revealed no significant difference between the glucose solution and the control containing only distilled water. When compared to the control, the COD (60 mg/L) seemed to be more effective at spore removal. Results however, were verified by using the Mann-Whitney U test which showed there was no significant difference ($p = 0.09$).

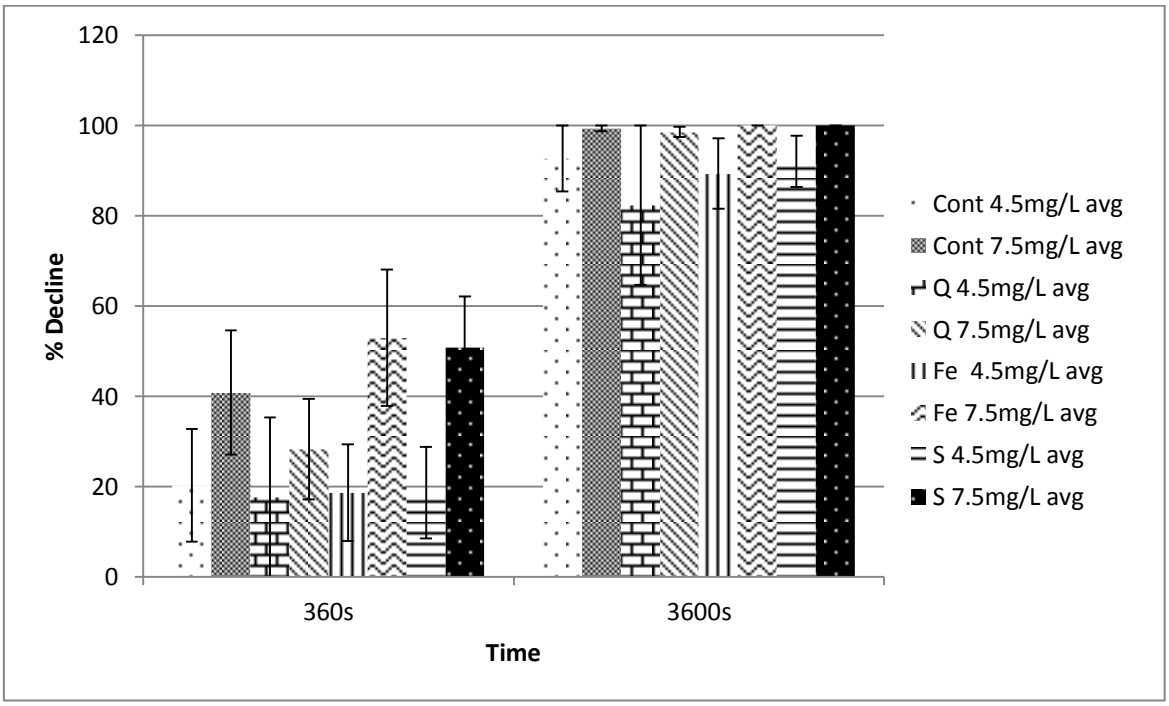

Figure 4.4. Decline of *Fusarium* spores treated with either 4.50 mg/L or 7.50 mg/L 'HTH® Super Shock It' in the presence of suspensions of quartz (Q), goethite (Fe), smectite (S). The control (Cont) contained distilled water without a mineral suspension. Measurements were conducted 360 s and 3600 s after treating the spores with the disinfectant. Values are the means of at least three repetitions, bars denote standard errors.

4.4 Conclusions

This study clearly showed that, at temperatures between 20°C and 23°C, 1.50 mg/L 'HTH® Super Shock It' will be effective at destroying *E. coli* and other faecal coliforms in Vaal River irrigation water, dispersed by a centre-pivot irrigation system. In contrast, the concentration of this calcium hypochlorite disinfectant needed to destroy *Fusarium* spores was notably higher.

The most effective concentration of 'HTH® Super Shock It' against *Fusarium* spores was 7.50 mg/L, as was observed after prolonged contact with the disinfectant (i.e. 360 s and 3600 s) however; nearly 90% of the spores survived after 16 s. This renders the use of 'HTH® Super Shock It', ineffective against *Fusarium* in a centre-pivot irrigation system in which the water is treated with chlorine 16 s before it is dispersed onto the crops. In addition, it must be noted that residual reactive chlorine still remained in the water one hour after treatment with 7.50 mg/L 'HTH® Super Shock It'. It is therefore recommended that, to minimise the risk of releasing excessive concentrations of residual chlorine onto crops via the irrigation system, at least one hour waiting period should be provided when the water is treated with these higher concentrations of 'HTH® Super Shock It'.

Similar to the findings of others, factors such as time, temperature, and pH impacted on the efficacy of 'HTH® Super Shock It' against *Fusarium* spores. In contrast to literature however, increased water turbidity was associated with an enhanced percentage decline of culturable *Fusarium* spores, in water treated with this chlorine containing disinfectant. No conclusive results were obtained in subsequent experiments when different mineral components of irrigation water were tested for the ability to enhance the killing effect of 'HTH® Super Shock It'. The influence of minerals on the efficacy of chlorine containing disinfectants should therefore be further studied. In addition, the effect of different concentrations of organic matter on the efficacy of HTH® Super Shock It' should also be studied.

Results of this study indicated that a centre-pivot irrigation system, in which water is treated with 'HTH® Super Shock It' for 16 s or longer before it is released onto the crops, might be effective for the removal of coliform bacteria from irrigation water originating from the Vaal River during summer when the water temperature reaches ca. 20 ºC. Nevertheless, the experment should be repeated at lower temperatures to confirm our observations. The system however, may not be effective in irradicating fungal plant pathogens, such as *Fusarium* from the water, and alternative methods of disinfection should be considered. Treating water with ozone or UV light technology (Edberg *et al*., 2000; WHO, 2008) may decrease the amount of spores in the water. Using crops resistant to plant pathogens (Collinge *et al*., 2010) may also minimise the incidents of infection by *Fusarium* spp.

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Addendum 1

Data pertaining to chlorine disinfection trials using *F. oxysporum* isolates collected during this study

Table 1. All data obtained in order to determine the effect time, temperature, pH and chlorine on the disinfection of *Fusarium oxysporum* spores.

[a] Strain	[HTH® Super Shock It]	Time in s	pH	Turb	Temp	Inoculum size (CFU/ml)	% Decline
F. oxysporum (CAB 157)	3.20 mg/L	16	8.63	23.80	20.90	250	0
F. oxysporum (CAB 157)	3.20 mg/L	16	8.66	48.30	21.40	250	28
F. oxysporum (CAB 157)	3.20 mg/L	16	8.46	45.80	21.70	280	0
F. oxysporum (CAB 157)	4.50 mg/L	16	8.36	43.60	21.80	280	7
F. oxysporum (CAB 157)	4.50 mg/L	16	8.60	19.70	22.40	470	34
F. oxysporum (CAB 157)	4.50 mg/L	16	8.72	24.00	22.00	240	0
F. oxysporum (CAB 157)	6.25 mg/L	16	8.35	26.00	22.00	230	0
F. oxysporum (CAB 157)	6.25 mg/L	16	8.59	40.00	22.30	270	0
F. oxysporum (CAB 157)	6.25 mg/L	16	8.76	22.50	22.30	260	4
F. oxysporum (CAB 157)	7.50 mg/L	16	8.67	46.50	21.90	290	21
F. oxysporum (CAB 157)	7.50 mg/L	16	8.82	11.75	22.00	240	0
F. oxysporum (CAB 157)	7.50 mg/L	16	8.55	52.20	22.80	260	23
F. oxysporum (CAB 157)	3.20 mg/L	360	8.63	23.80	20.90	250	0
F. oxysporum (CAB 157)	3.20 mg/L	360	8.66	48.30	21.40	250	0
F. oxysporum (CAB 157)	3.20 mg/L	360	8.46	45.80	21.70	280	11
F. oxysporum (CAB 157)	4.50 mg/L	360	8.36	43.60	21.80	280	25
F. oxysporum (CAB 157)	4.50 mg/L	360	8.60	19.70	22.40	470	19
F. oxysporum (CAB 157)	4.50 mg/L	360	8.72	24.00	22.00	240	0
F. oxysporum (CAB 157)	6.25 mg/L	360	8.35	26.00	22.00	230	48
F. oxysporum (CAB 157)	6.25 mg/L	360	8.59	40.00	22.30	270	100

[a] Strain	[HTH® Super Shock It]	Time in s	pH	Turb	Temp	Inoculum size (CFU/ml)	% Decline
F. oxysporum (CAB 157)	6.25 mg/L	360	8.76	22.50	22.30	260	62
F. oxysporum (CAB 157)	7.50 mg/L	360	8.67	46.50	21.90	290	100
F. oxysporum (CAB 157)	7.50 mg/L	360	8.82	11.75	22.00	240	71
F. oxysporum (CAB 157)	7.50 mg/L	360	8.55	52.20	22.80	260	100
F. oxysporum (CAB 157)	3.20 mg/L	3600	8.63	23.80	20.90	250	90
F. oxysporum (CAB 157)	3.20 mg/L	3600	8.66	48.30	21.40	250	67
F. oxysporum (CAB 157)	3.20 mg/L	3600	8.46	45.80	21.70	280	96
F. oxysporum (CAB 157)	4.50 mg/L	3600	8.36	43.60	21.80	280	100
F. oxysporum (CAB 157)	4.50 mg/L	3600	8.60	19.70	22.40	470	100
F. oxysporum (CAB 157)	4.50 mg/L	3600	8.72	24.00	22.00	240	100
F. oxysporum (CAB 157)	6.25 mg/L	3600	8.35	26.00	22.00	230	100
F. oxysporum (CAB 157)	6.25 mg/L	3600	8.59	40.00	22.30	270	100
F. oxysporum (CAB 157)	6.25 mg/L	3600	8.76	22.50	22.30	260	100
F. oxysporum (CAB 157)	7.50 mg/L	3600	8.67	46.50	21.90	290	100
F. oxysporum (CAB 157)	7.50 mg/L	3600	8.82	11.75	22.00	240	100
F. oxysporum (CAB 157)	7.50 mg/L	3600	8.55	52.20	22.80	260	100
F. oxysporum (CAB 154)	3.20 mg/L	16	8.85	29.70	23.20	280	43
F. oxysporum (CAB 154)	3.20 mg/L	16	9.14	26.00	22.30	250	4
F. oxysporum (CAB 154)	3.20 mg/L	16	8.93	44.10	22.40	230	22
F. oxysporum (CAB 154)	4.50 mg/L	16	9.48	16.62	21.80	180	0
F. oxysporum (CAB 154)	4.50 mg/L	16	9.05	25.30	22.10	160	0

[a] Strain	[HTH® Super Shock It]	Time in s	pH	Turb	Temp	Inoculum size (CFU/ml)	% Decline
F. oxysporum (CAB 154)	4.50 mg/L	16	9.11	27.00	22.80	200	65
F. oxysporum (CAB 154)	6.25 mg/L	16	9.10	28.70	21.90	120	8
F. oxysporum (CAB 154)	6.25 mg/L	16	9.30	36.90	22.00	100	0
F. oxysporum (CAB 154)	6.25 mg/L	16	9.47	17.76	22.50	150	0
F. oxysporum (CAB 154)	7.50 mg/L	16	9.05	27.90	21.50	160	6
F. oxysporum (CAB 154)	7.50 mg/L	16	9.14	22.30	21.60	150	33
F. oxysporum (CAB 154)	7.50 mg/L	16	9.17	27.40	22.10	120	8
F. oxysporum (CAB 154)	3.20 mg/L	360	8.85	29.70	23.20	280	36
F. oxysporum (CAB 154)	3.20 mg/L	360	9.14	26.00	22.30	250	32
F. oxysporum (CAB 154)	3.20 mg/L	360	8.93	44.10	22.40	230	74
F. oxysporum (CAB 154)	4.50 mg/L	360	9.48	16.62	21.80	180	0
F. oxysporum (CAB 154)	4.50 mg/L	360	9.05	25.30	22.10	160	25
F. oxysporum (CAB 154)	4.50 mg/L	360	9.11	27.00	22.80	200	0
F. oxysporum (CAB 154)	6.25 mg/L	360	9.10	28.70	21.90	120	0
F. oxysporum (CAB 154)	6.25 mg/L	360	9.30	36.90	22.00	100	0
F. oxysporum (CAB 154)	6.25 mg/L	360	9.47	17.76	22.50	150	20
F. oxysporum (CAB 154)	7.50 mg/L	360	9.05	27.90	21.50	160	100
F. oxysporum (CAB 154)	7.50 mg/L	360	9.14	22.30	21.60	150	80
F. oxysporum (CAB 154)	7.50 mg/L	360	9.17	27.40	22.10	120	67
F. oxysporum (CAB 154)	3.20 mg/L	3600	8.85	29.70	23.20	280	100
F. oxysporum (CAB 154)	3.20 mg/L	3600	9.14	26.00	22.30	250	100

[a] Strain	[HTH® Super Shock It]	Time in s	pH	Turb	Temp	Inoculum size (CFU/ml)	% Decline
F. oxysporum (CAB 154)	3.20 mg/L	3600	8.93	44.10	22.40	230	100
F. oxysporum (CAB 154)	4.50 mg/L	3600	9.48	16.62	21.80	180	100
F. oxysporum (CAB 154)	4.50 mg/L	3600	9.05	25.30	22.10	160	100
F. oxysporum (CAB 154)	4.50 mg/L	3600	9.11	27.00	22.80	200	100
F. oxysporum (CAB 154)	6.25 mg/L	3600	9.10	28.70	21.90	120	100
F. oxysporum (CAB 154)	6.25 mg/L	3600	9.30	36.90	22.00	100	100
F. oxysporum (CAB 154)	6.25 mg/L	3600	9.47	17.76	22.50	150	100
F. oxysporum (CAB 154)	7.5 mg/L	3600	9.05	27.90	21.50	160	100
F. oxysporum (CAB 154)	7.5 mg/L	3600	9.14	22.30	21.60	150	100
F. oxysporum (CAB 154)	7.5 mg/L	3600	9.17	27.40	22.10	120	100
F. oxysporum (CAB 156)	3.20 mg/L	16	8.59	50.80	22.30	450	0
F. oxysporum (CAB 156)	3.20 mg/L	16	8.87	25.70	22.30	340	0
F. oxysporum (CAB 156)	3.20 mg/L	16	8.69	58.90	22.40	380	0
F. oxysporum (CAB 156)	4.50 mg/L	16	8.77	80.00	22.70	520	13
F. oxysporum (CAB 156)	4.50 mg/L	16	8.96	26.20	22.30	340	0
F. oxysporum (CAB 156)	4.50 mg/L	16	9.06	28.20	22.70	320	0
F. oxysporum (CAB 156)	6.25 mg/L	16	8.52	83.40	23.10	460	9
F. oxysporum (CAB 156)	6.25 mg/L	16	8.52	87.00	22.90	360	14
F. oxysporum (CAB 156)	6.25 mg/L	16	8.64	80.90	22.50	400	3
F. oxysporum (CAB 156)	7.50 mg/L	16	8.86	75.90	22.60	390	0
F. oxysporum (CAB 156)	7.50 mg/L	16	8.75	64.90	23.00	330	0

[a] Strain	[HTH® Super Shock It]	Time in s	pH	Turb	Temp	Inoculum size (CFU/ml)	% Decline
F. oxysporum (CAB 156)	7.50 mg/L	16	8.66	78.20	23.00	490	16
F. oxysporum (CAB 156)	3.20 mg/L	360	8.59	50.80	22.30	450	29
F. oxysporum (CAB 156)	3.20 mg/L	360	8.87	25.70	22.30	340	0
F. oxysporum (CAB 156)	3.20 mg/L	360	8.69	58.90	22.40	380	29
F. oxysporum (CAB 156)	4.50 mg/L	360	8.77	80.00	22.70	520	56
F. oxysporum (CAB 156)	4.50 mg/L	360	8.96	26.20	22.30	340	9
F. oxysporum (CAB 156)	4.50 mg/L	360	9.06	28.20	22.70	320	0
F. oxysporum (CAB 156)	6.25 mg/L	360	8.52	83.40	23.10	460	87
F. oxysporum (CAB 156)	6.25 mg/L	360	8.52	87.00	22.90	360	94
F. oxysporum (CAB 156)	6.25 mg/L	360	8.64	80.90	22.50	400	88
F. oxysporum (CAB 156)	7.50 mg/L	360	8.86	75.90	22.60	390	90
F. oxysporum (CAB 156)	7.50 mg/L	360	8.75	64.90	23.00	330	97
F. oxysporum (CAB 156)	7.50 mg/L	360	8.66	78.20	23.00	490	98
F. oxysporum (CAB 156)	3.20 mg/L	3600	8.59	50.80	22.30	450	100
F. oxysporum (CAB 156)	3.20 mg/L	3600	8.87	25.70	22.30	340	100
F. oxysporum (CAB 156)	3.20 mg/L	3600	8.69	58.90	22.40	380	100
F. oxysporum (CAB 156)	4.50 mg/L	3600	8.77	80.00	22.70	520	100
F. oxysporum (CAB 156)	4.50 mg/L	3600	8.96	26.20	22.30	340	100
F. oxysporum (CAB 156)	4.50 mg/L	3600	9.06	28.20	22.70	320	100
F. oxysporum (CAB 156)	6.25 mg/L	3600	8.52	83.40	23.10	460	100
F. oxysporum (CAB 156)	6.25 mg/L	3600	8.52	87.00	22.90	360	100

[a] Strain	[HTH® Super Shock It]	Time in s	pH	Turb	Temp	Inoculum size (CFU/ml)	% Decline
F. oxysporum (CAB 156)	6.25 mg/L	3600	8.64	80.90	22.50	400	100
F. oxysporum (CAB 156)	7.50 mg/L	3600	8.86	75.90	22.60	390	100
F. oxysporum (CAB 156)	7.50 mg/L	3600	8.75	64.90	23.00	330	100
F. oxysporum (CAB 156)	7.50 mg/L	3600	8.66	78.20	23.00	490	100
F. oxysporum (CAB 158)	3.20 mg/L	16	8.77	36.70	n.d	680	15
F. oxysporum (CAB 158)	3.20 mg/L	16	8.92	193.00	n.d	350	19
F. oxysporum (CAB 158)	3.20 mg/L	16	9.00	58.70	n.d	380	37
F. oxysporum (CAB 158)	6.25 mg/L	16	9.13	22.80	n.d	490	6
F. oxysporum (CAB 158)	6.25 mg/L	16	8.86	43.60	n.d	490	24
F. oxysporum (CAB 158)	6.25 mg/L	16	8.86	38.10	n.d	450	0
F. oxysporum (CAB 158)	3.20 mg/L	360	8.77	36.70	n.d	680	28
F. oxysporum (CAB 158)	3.20 mg/L	360	8.92	193.00	n.d	350	33
F. oxysporum (CAB 158)	3.20 mg/L	360	9.00	58.70	n.d	380	0
F. oxysporum (CAB 158)	6.25 mg/L	360	9.13	22.80	n.d	490	10
F. oxysporum (CAB 158)	6.25 mg/L	360	8.86	43.60	n.d	490	33
F. oxysporum (CAB 158)	6.25 mg/L	360	8.86	38.10	n.d	450	20
F. oxysporum (CAB 158)	3.20 mg/L	3600	8.77	36.70	n.d	680	81
F. oxysporum (CAB 158)	3.20 mg/L	3600	8.92	193.00	n.d	350	80
F. oxysporum (CAB 158)	3.20 mg/L	3600	9.00	58.70	n.d	380	100
F. oxysporum (CAB 158)	6.25 mg/L	3600	9.13	22.80	n.d	490	100
F. oxysporum (CAB 158)	6.25 mg/L	3600	8.86	43.60	n.d	490	100

[a] Strain	[HTH® Super Shock It]	Time in s	pH	Turb	Temp	Inoculum size (CFU/ml)	% Decline
F. oxysporum (CAB 158)	6.25 mg/L	3600	8.86	38.10	n.d	450	100

[a] CAB numbers of strains used, collected from Vaal River water and onions from the Vaal region were deposited in the fungal culture collection of Stellenbosch University, Department Microbiology

n.d, denotes, not determined

[HTH® Super Shock It], refers to the concentration of 'HTH® Super Shock It' added

Turb, denotes, Turbidity

Temp, denotes, Temperature

Chapter 5

General conclusions and future research

5.1 General conclusion

Water from the Vaal River is an important asset to South Africa and its people. The river spans three water management areas (Upper, Middle, and Lower Vaal), supporting activities such as mining, industry, dry land cultivation, livestock farming, as well as intensive vegetable production (DWAF, 2004c; DWAF, 2009). The latter production systems occur in the Lower Vaal where large scale onion production takes place, utilising centre-pivot irrigation. Worryingly, degradation of the Vaal River as a water source has been observed, and concerns were raised by local farmers who use the Vaal River to irrigate their crops (DWA, 2011). These concerns include sewage polluted river water and the concommitant potential transfer of waterborne human pathogens to crops (Steele & Odumeru, 2004; Steele *et al.,* 2005; Tempelhoff, 2009; Alsanius *et al*., 2010). Also, agricultural surface runoff ending up in rivers, may contain waterborne plant pathogens which could pose a threat to production when introduced to crops via irrigation (Bucheli *et al.,* 2008; Summerell *et al.*, 2010; Van Wyk *et al*., 2012). Despite these concerns, knowledge of the impact of waterborne fungi on water quality, and the treatment of fungal pollutants, is lacking and rarely reported on (Hageskal *et al*., 2009). Thus, the overall goals of this study were firstly to screen for faecal contamination in the water of the Lower Vaal, as well as to determine whether irrigation water from the Vaal River contained the onion pathogen *Fusarium oxysporum* f. sp. *cepae* (*Focep*). Lastly the efficacy of a cost effective disinfectant, i.e. calcium hypochlorite, was determined, in the removal of coliform bacteria and *Fusarium* spores from river water.

Surface water samples, as well as irrigation water from various pump stations, were collected at sampling sites along a 159 km stretch of the Vaal River, in the Lower Vaal management area situated in the Northern Cape. Analyses of the 63 water samples collected over a period of three years revealed that faecal coliform concentrations, as enumerated on MacConkey agar were always present in the river. The occurrence of faecal coliforms, indicative of bacterial pathogens, such as, *Escherichia coli, Campylobacter jejuni, Salmonella* spp, *Shigella* spp, and *Vibrio cholerae,* was confirmed when the human enteric pathogen, *Escherichia coli* 0157:H7 was detected in a water sample using the 3M™ Molecular Detection System.

Inoculation of potato dextrose agar with filtrates of 73 water samples collected during the same period, and from the same stretch of the Vaal River, as mentioned above, yielded a total of 59 single-spore *Fusarium* isolates. During the same period, eight *Fusarium oxysporum* isolates were acquired from onion bulbs (Lombardi cultivar) cultivated in the same region, (one symptomatic showing basal rot, and seven asymptomatic bulbs) irrigated with water from the Vaal River.

Subsequent molecular identification confirmed that the isolates belonged to four *Fusarium* species, *Fusarium brachygibbosum, Fusarium incarnatum-equiseti, Fusarium solani* and *Fusarium oxysporum,* the latter being the dominant species represented by 52 isolates.

Even though the Vaal River irrigation water contained several *Fusarium* species, none of these were pathogenic towards the Lombardi onion cultivar and were therefore not representatives of the onion pathogen, *Focep* (Schwartz & Mohan, 2008). However, four *Fusarium oxysporum* isolates obtained from onion bulbs (CAB 152, 155, 156, and 158) were pathogenic towards the Lombardi onion cultivar and were classified as *Focep.* Subsequent determination of the vegetative compatibility of these *Focep* isolates revealed they belonged to the vegetative compatibility group (VCG) 0425. This was the second documented incidence of VCG 0425 in the Northern Cape area (Philippou, 2014). Previously it was reported that VCG 0425 resulted in severe onion losses in the Western Cape Province (Southwood *et al*., 2012). This suggested that VCG 0425 may have been introduced to the Northern Cape by means other than irrigation water, such as infected seedlings from the Western Cape.

Despite the fact that *Fusarium* isolates from the Vaal River caused no disease to onion. Screening the waterborne *Fusarium* isolates for *SIX* (secreted in xylem) genes revealed that a number of these isolates contain virulence factors. The non-pathogenic isolates CAB 143, 144, 153, 164, 331 and 350 were shown to harbour the *SIX7* gene making this the second known study to demonstrate that *Focep* isolates contain *SIX* genes capable of producing virulence factors. Therefore, other produce cultivated in the Lower Vaal area, may still be at risk of infection from *Fusarium oxysporum* strains harbouring the *SIX7* gene.

The potential presence of both human and plant pathogens in water from the Vaal River lead to the recommendation that the water should be treated with a disinfectant to reduce pathogen levels before it is used to irrigate crops. The objective of the final part of the study was therefore to evaluate the efficacy of a calcium hypochlorite containing disinfectant ('HTH® Super Shock It') to remove faecal coliforms and *Fusarium* strains from Vaal River irrigation water. A low concentration (1.50 mg/L) of the disinfectant was required to effectively remove faecal coliforms at a contact time of 16 s. In contrast, both a higher calcium hypochlorite concentration (7.50 mg/L) and longer contact time (3600 s) was needed to remove *Fusarium* spores from the water. In practise, the longer contact time would render the disinfectant ineffective at removing fungal plant pathogens from a centre-pivot irrigation system, where water is treated for 16 s before being dispersed onto crops. High chlorine residuals however, remained after 16 s of contact time when the initial concentration of 'HTH® Super Shock It' was 4.50 mg/L or 7.50 mg/L. To minimise the risk of damaging crops when using higher disinfectant concentrations a one-hour waiting period was recommended before the chlorine treated water is dispersed onto the crops.

In addition to time, factors such as temperature and pH affected the efficacy of the disinfectant in removing *Fusarium* spores. A positive correlation was also discovered between increased water turbidity and the removal of *Fusarium* spores.

Subsequent experiments to assess this observation were conducted by evaluating organic and inorganic composition, as well as the different mineral components of the river water. However, no conclusive results were obtained. In future, more experiments have to be conducted in order to better understand the interaction between water turbidity and the removal of *Fusarium* spores using 'HTH® Super Shock It'.

To conclude, it is clear from the study that the Lower Vaal River is contaminated with faecal pollution, which may taint crops via irrigation. Waterborne plant pathogens are also a potential risk to crops seeing that certain virulence factors were observed in a number of Vaal River *F. oxysporum* isolates. In future, pathogenicity tests should also be conducted on crops other than onion cultivated in the region, since these isolates may be pathogenic towards a different crop. Additionally, more *Fusarium* isolates must be collected from the Lower Vaal River, and used in pathogenicity studies. This should be done to confirm the absence of *Focep* in the river water and to determine whether other potential plant pathogenic *Fusarium* species occur in the river.

Furthermore, this study has shown that faecal coliforms in river water could successfully be treated with a calcium hypochlorite containing disinfectant within the operating parameters of a centre-pivot irrigation system. This disinfectant may not be as effective in removing *Fusarium* spores however; other disinfection practices can be employed. Treatment with ozone or UV light technology may remove fungal spores from the water (Edberg *et al*., 2000; WHO, 2008). Crops resistant to plant pathogens could also minimise the incidents of infection by *Fusarium* spp (Collinge *et al*., 2010).

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